The Assessment of a Minimally Invasive Procedure in the Treatment of Deep Carious Lesions
In Vivo and In Vitro Studies

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The Assessment of a Minimally Invasive Procedure in the Treatment of Deep Carious Lesions: *In Vivo* and *In Vitro* Studies

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Abstract

Aims and objectives: the aim of this dissertation was to determine the effectiveness of a minimally invasive indirect clinical pulp protection technique in preserving pulp vitality in symptomatic teeth *in vivo*. The objectives were firstly to assess the *in-vitro* characteristics and mechanical behaviour of the interfaces between MTA and the overlying restorations of GIC and resin composite. The second objective was to assess the effect of each excavation technique (rotary drill versus Carisolv^{TM} gel) in promoting the remineralization of caries-affected dentine capped with mineral trioxide aggregate in deep carious teeth *in-vitro*. The third objective was to compare clinically and radiographically the one year outcomes of indirect pulp protection performed using mineral trioxide aggregate (MTA) in teeth with deep carious lesions excavated using a minimally invasive clinical protocol (Carisolv^{TM} gel chemomechanical system and an operating microscope) with a control protocol (rotary burs with no magnification) in patients presenting with signs and symptoms of reversible pulpitis. The fourth objective was to determine the reduction in bacterial load before and after excavation with the two clinical protocols and to identify the bacterial flora composition / diversity in superficial and deep carious dentine samples within teeth included in the clinical trial. The third objective was

Materials and methods: The *in-vitro* study involved shear bond strength testing and SEM fractography of MTA discs bonded to RC with an etch and rinse adhesive or conventional GIC after 10-minute, 24-hour, 72-hour and 30-day of MTA setting intervals. The other *in-vitro* study involved using Raman spectroscopy and Knoop microhardness of the MTA/dentine interface at 24 hours and 14 days intervals in cavities of teeth excavated either with rotary bur or Carisolv^{TM} gel. In addition, SEM was used to assess surface topography of dentine after excavation with gel/bur.

A one year duration, randomised controlled clinical trial was undertaken which involved recruiting patients with signs of reversible pulpitis from King's College London Dental Institute at Guy's Hospital. Standardised clinical and radiographic investigations involving CBCT and PA radiographs at baseline and one-year follow-up were used to assess pulp pathology (presence/absence of PA radiolucencies). Caries was excavated conventionally using rotary burs/no magnification in the control group and Carisolv™ gel/operating microscope in the minimally invasive group. Each tooth received an indirect pulp capping with MTA followed by an intermediate layer of GIC before placing a resin composite (RC) restoration in one visit. In addition, bacterial load and composition of the bacterial flora were assessed using non-culture-based DNA extraction, qPCR and sequencing of the 16S rRNA gene by using next-generation high throughput sequencing.

Result: *In-vitro* studies showed higher shear bond strength of RC to MTA after 24hr, 7 and 30 days compared to that of GIC to MTA which was higher in the initial 10 minutes setting group. Also baseline mineral content and microhardness of dentine were significantly lower in samples excavated with Carisolv™ gel in comparison to those excavated with rotary burs ($p \leq 0.05$). However, there were comparable mineral levels after 14 days between them after storage in simulated body fluid ($p \geq 0.05$), SEM images showed partially open dentine tubules with less smear layer after Carisolv™ gel excavation compared to more occluded dentine tubules with an abundance of smear layer after rotary bur excavation. Results from the clinical trial show 101 restorations (55 and 46 restorations in control and minimally invasive groups respectively) were placed randomly in 86 patients as part of the clinical trial. Success rates were 73.3% and 90% in the control and the minimally invasive groups, respectively. The minimally invasive protocol in molar teeth showed a higher odds ratio of success compared to the

control protocol and premolars, respectively. CBCT detected more PA lesions than PA radiographs. Teeth with “severe” reversible pulpitis symptoms lost vitality more often than teeth with “mild” symptoms. Microbiological analysis showed no significant difference in reduction of bacterial load between the minimally invasive technique and the control protocol. *Lactobacillus* was the most abundant genus in the superficial and deep caries samples and there was no significant difference in bacterial composition between superficial and deep carious dentine.

Conclusions: Placement of RC over partially set MTA in one visit vital pulp therapy avoided considering the low bond strength values achieved after 10 min from placement. Although Carisolv^{TM} gel excavation retains more caries-affected dentine in comparison to bur excavation, remineralisation of this remaining tissue is evident after two weeks. The use of Carisolv^{TM} gel provides an alternative to rotary burs in terms of preserving tooth structure and ability to provide dentine remineralization, a clinical advantage in minimally invasive operative dentistry. The minimally invasive protocol was more effective in preserving pulp vitality in teeth with reversible pulpitis compared to the control protocol. The minimally invasive protocol was able to reduce bacterial numbers in dentine caries after excavation as well as the control protocol. There was no significant difference in bacterial composition between superficial and deep carious dentine samples. However, it was noted that carious lesions can be classified according to the relative abundance of *Lactobacillus* species. Carious lesions with low-*Lactobacillus* abundance were dominated by other bacterial taxa which were frequently isolated from root canal infections.

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List of Abbreviations

CBCT: cone-beam computed tomography

In-vivo: within living organisms.

IPC: indirect pulp capping

In-vitro: in an artificial environment outside the living organism

MTA: mineral trioxide aggregate

GIC: glass ionomer cement

EDJ: enamel-dentine junction

BMP: bone morphogenic protein

DSP: dentine sialoprotein

DPP: dentine phosphoprotein

DMP: dentine matrix protein

TGF-β: transforming growth factor-β

DSPP: Dentine sialophosphoprotein

MMPs: Matrix metalloproteinases

MT1-MMP: membrane type-1 matrix metalloproteinase

TIMP-2: metalloproteinase-2

TLR-4:

NF-κB: nucleic factor kappa B

IL-1a: interleukin-1a

TNF-a: tumour necrosis factor-a

AP-1: activator protein-1

TEGDMA: triethylene glycol dimethacrylate

HEMA: hydroxyethyl methacrylate

RMGIC: resin modified glass ionomer cement

ZOE: zinc oxide eugenol

EPT: electric pulp testing

CT: computed tomography

NaOCl: sodium hypochlorite

SEM: scanning electron microscope

Ca:P : Calcium/phosphate ratio

ECC: childhood dental caries

spp.: species

PCR: polymerase chain reaction

RC: resin composite

IFM: interfacial failure mode

Mpa: megapascal

SBS: shear bond strength

CAD: caries-affected dentine

VHN: Vicker hardness number

DW: distilled water

SBF: simulated body fluid

MPI: mineral peak intensity

$PO4^{-3}$: phosphate group

OCP: octa-calcium phosphate

DCPD: dicalcium phosphate dihydrate

TCP: tricalcium phosphate

ACP: amorphous calcium phosphate

T0: baseline visit

T12: one-year follow-up visit

RCT: Randomised Controlled Clinical Trial

GSTFT: Guy's St Thomas foundation trust

R&D: research and development

PDL: periodontal ligament

FAM: carboxyfluorescein

TAMRA: tetramethylrhodamine

NGS: Next generation sequencing

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In the name of “Allah” the greatest and the most merciful, to whom all thanks and praises for providing me with strength and patience to complete this work and journey.

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Chapter 1 Introduction

1.1 Overview

The diagnosis of reversible pulp inflammation is important to predict the outcome of restorative procedures, however, such diagnosis is still a challenge due to the imprecise representation of the histological status of the pulp associated with the current clinical and radiographical diagnostic means (Dummer *et al.* 1980). Shortcomings of two-dimensional periapical radiographs, which include dimensional distortion, anatomical noise and superimposition, can affect diagnosis (Patel *et al.* 2007). These can be partially overcome with the use of three-dimensional cone-beam computed tomography (CBCT).

Indirect pulp capping can provide a successful modality for the treatment of deep carious lesions in both primary and permanent teeth and it is able to prevent further progression of carious lesions regardless of the type of the pulp capping materials used (Al-Zayer *et al.* 2003, Marchi JJ *et al.* 2009, Hashem *et al.* 2015). The conventional way of treatment of deep carious lesions by removing all carious dentine may lead to iatrogenic pulp exposure and subsequent root canal treatment compared with partial caries removal, which is based on understanding the caries process and defence reactions of the dentine-pulp complex (Kidd 2010), therefore the focus on exploiting a new conservative deep caries management technique with predictable outcome will be the focus of the current research.

1.2 Research aims and objectives

This research project is divided into two parts:

The first part is an *in-vivo* study in the form of a single-blinded randomised controlled *clinical trial* to evaluate primarily the effect of two caries excavation protocols (a conventional approach using rotary burs versus a minimally invasive approach carried out with the use of an endodontic microscope and Carisolv^{TM} gel) in patients with signs and symptoms of reversible pulpitis using mineral trioxide aggregate as a indirect pulp-capping agent, the study also assessed the effectiveness of each procedure on the change of diversity and infectivity of bacteria associated with deep caries.

The second arm is composed of *in-vitro* investigations to support the clinical arm, firstly to assess the interfacial characteristics between mineral trioxide aggregate (MTA) and the overlying restorations. Secondly to assess the effect of both excavation techniques on the remineralization of caries-affected dentine capped with mineral trioxide aggregate *in-vitro*.

The general aims of the project are to establish whether a more conservative indirect pulp capping procedure undertaken using an operative microscope, a chemo-mechanical caries removal method and MTA is able to improve the preservation of vitality of teeth with deep caries in patients with signs/symptoms of reversible pulpitis.

The objectives of the study were the following:

1. Assess the clinical and radiographical success/failure of teeth treated with two different techniques of indirect pulp capping; follow-up was at 12 months.
2. Assess the bacterial composition and the number of bacteria in carious dentine samples taken before and after each clinical excavation technique using qPCR and 16S rRNA next generation high throughput sequencing.

3. Assess the effectiveness of CBCT scans in the detection of periapical lesions compared to conventional radiographs in teeth with signs and symptoms of reversible pulpitis.
4. Assess the mechanical characteristics of the interface between MTA as a pulp capping agent and overlying restoratives materials (*in-vitro*).
5. Assess dentine remineralisation after dentinal caries excavation either with the chemomechanical or rotary burs caries removal methods (*in-vitro*).

1.3 Structure of the thesis

This thesis is divided into six chapters as shown in Figure 1-1; the first chapter gives a brief overview of aims, objectives and structure of the research. The second chapter provides a critical review of literature of dental caries and the response of dentine-pulp complex to dental caries. Dentinogenesis and responses of dentine-pulp complex to both restorative procedures and restorative materials are also discussed. Indirect pulp capping clinical approaches are reviewed, this is followed by a description of the current different caries removal methods with a focus on the minimal invasive approach. The last section of the literature review deals with chemical, mechanical and clinical characteristics of the materials used for pulp capping including calcium hydroxide and MTA. The last section describes the microbiological aspects of dental caries from a molecular point of view. Components of literature review are illustrated in Figure 2-1

Chapter three include the first *in-vitro* experiment to assess the mechanical characteristics of the interface between MTA and restoratives materials including GIC and resin composite. Chapter four is dedicated to the second *in-vitro* experiment to assess the remineralisation potential of the remaining caries-affected dentine after excavation with Carisolv^{TM} or rotary burs methods of caries removal in deep carious lesions. Chapter five is a randomised controlled clinical trial to assess a minimally invasive caries excavation protocol including the use of Carisolv^{TM} gel and operative microscope compared to the traditional approach of caries excavation which utilises rotary burs and does not involve the magnification offered by the operative microscope for deep caries removal in patients with signs and symptoms of reversible pulpitis. Patients were followed up clinically and radiographically using periapical radiographs and Cone Beam Computed Tomography (CBCT) for 1-year after treatment. In addition, a molecular microbiological analysis was conducted (Chapter 6) to assess the difference between the techniques in terms of change in the composition of the remaining bacterial biofilm after each excavation procedure . Chapter seven includes a general summary of the outcomes of the results of the four studies together with suggestions for future research.

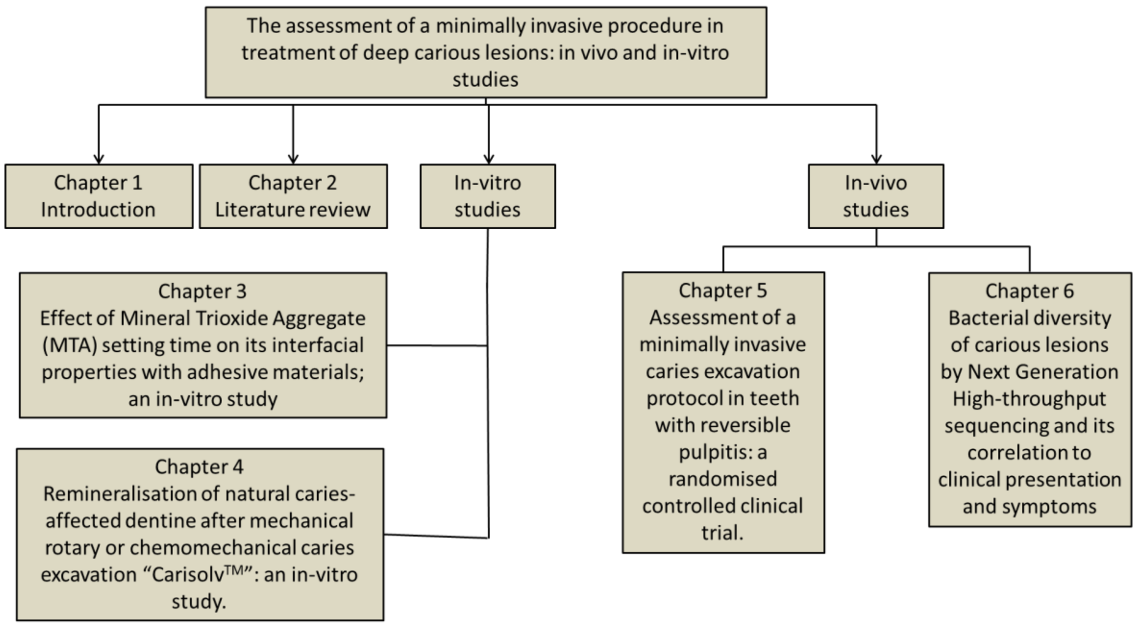


Figure 1-1: Structure of the PhD project

Chapter 2 Literature Review

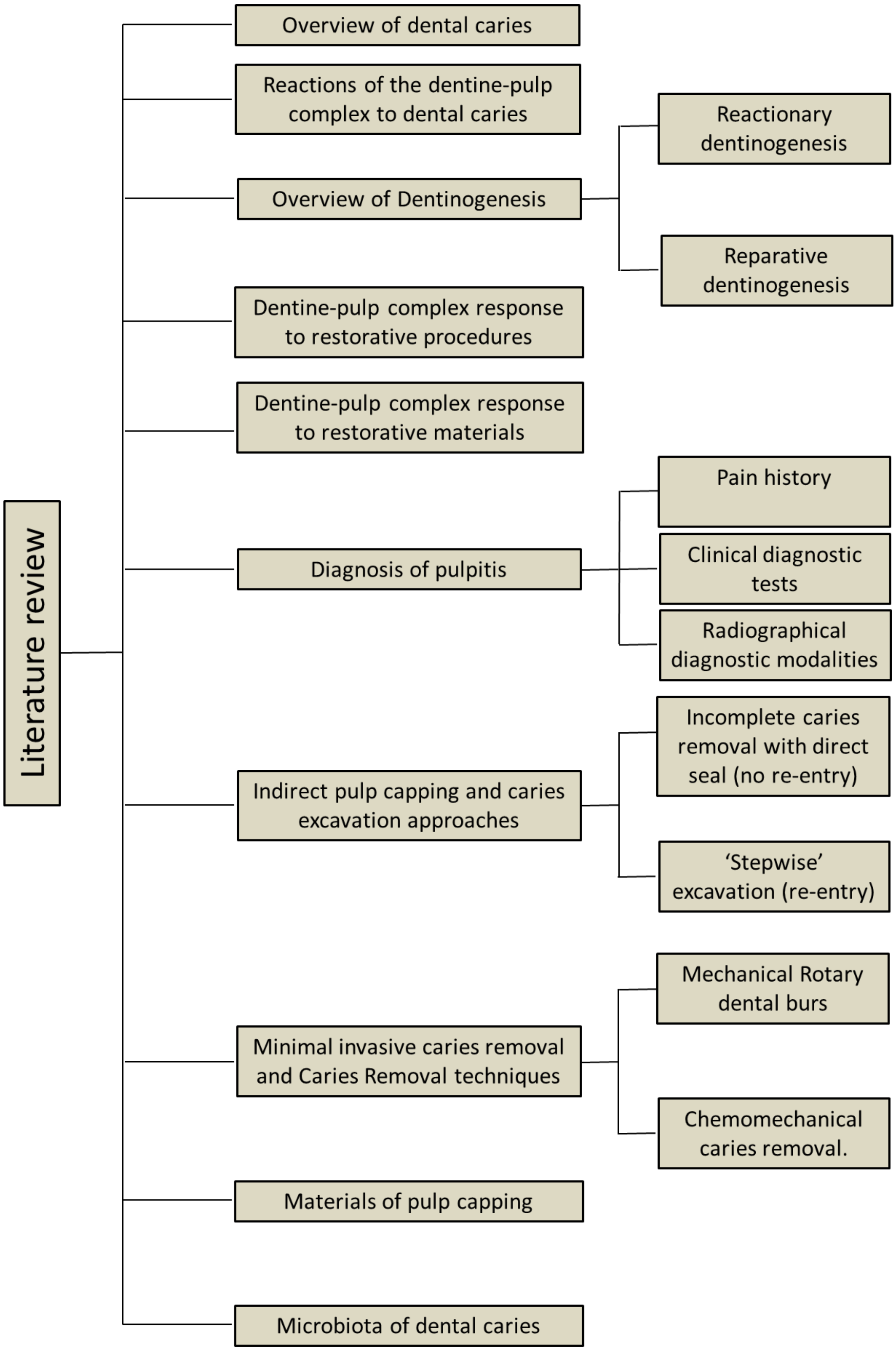


Figure 2-1: Components of literature review

2.1 Overview of dental caries

Caries still represents one of the most widespread chronic human diseases (Langeland 1987, Selwitz *et al.* 2007). It has been described as a transmissible and infectious disease (Heymann *et al.* 2014) and It is considered as the main cause of oral pain and tooth loss (Kidd *et al.* 2000). The interaction of the biofilm with the tooth surface may result in the formation of caries if other factors create the correct local environment (Kidd 2010). The caries process involves active de- and remineralisation processes in the tooth structure which results from a pH shift of biofilm due to products of microbial metabolism which over time may result in a net loss of mineral, and subsequently may lead to cavitation (Fejerskov 1997, Heymann *et al.* 2014). The process of demineralisation and remineralisation is affected by many factors which include type and number of bacteria in the biofilm, genetics, diet, oral hygiene, use of fluoride, salivary flow and composition of tooth (Heymann *et al.* 2014)

Caries extensively destroys the tissues of the tooth including dental pulp; caries can cause inflammation or sensitivity due to inflammation of pulpal tissue, which may become irreversible if it is left untreated and might lead to an infection and subsequent dental abscess. The process of caries development and progression is reversible and can be arrested in its early stages (Fejerskov and Kidd 2009).

Caries in enamel (enamel originates from the ectodermal part of the tooth germ) presents in its early stage as a non-cavitated white spot lesion, usually on the facial or lingual surfaces of the teeth. It is usually associated with smooth surfaces undetectable by dental explorer; more advanced lesion develops rough surface texture a sign of active lesion. Remineralised or arrested lesions of enamel can be observed as intact brown or black spots on enamel surface (Heymann *et al.* 2014). Dentino-pulpal complex responds to enamel caries by sclerosis (translucent zone), which is peritubular dentine precipitation alongside with crystals precipitation in the lumen of dentinal tubules (Fejerskov and Kidd 2008).

Dentine has a tubular structure, originates from the mesenchymal part of the tooth germ and contains fewer minerals compared to enamel. Caries in dentine is characterised by a brownish discoloration and once the enamel lesion extends past the enamel-dentine junction (EDJ), which is the least resistant to caries attack, the lateral lesion spread is confined by the size of the enamel lesion in contact with the EDJ, indicating a reaction to the biofilm activity on the enamel surface (Kidd and Fejerskov 2004, Heymann *et al.* 2014). Because of the action of acid and proteolytic enzymes, decomposition of the most superficial part of dentine occur and is called “zone of destruction”, followed by a zone of bacterial invasion into the dentinal tubules. Between the bacterial invasion zone and the zone of sclerosis, there is a zone of demineralisation due to by-products of bacteria in the cavity (Kidd and Fejerskov 2004). Figure 2-2 shows a diagrammatic representation of a carious lesion in dentine.

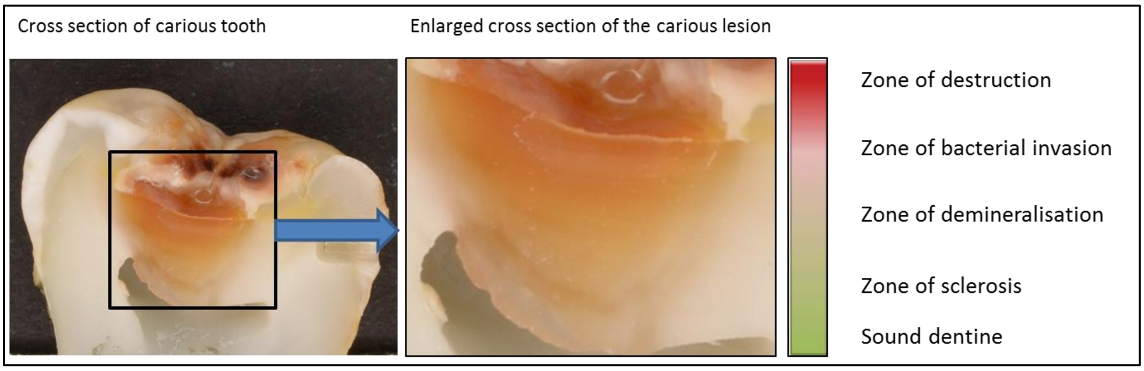


Figure 2-2: Diagram of dentine carious lesion.

The main two layers of carious dentine are the outer caries-infected and the inner caries-affected dentine layers (Fusayama and Terachima 1972). These two layers cannot be separated histologically. The caries-infected layer appears clinically as a dark, brown and soft layer, considered a non-repairable necrotic area (A. Banerjee *et al.* 1999). The inner layer of caries-affected dentine, which is the most frequently involved layer in clinical practice (Wang *et al.* 2007), is described clinically as sticky, harder, paler brown dentine and histologically can be divided into three zones: the turbid zone, transparent zone and sub-transparent zone (Fusayama *et al.* 1979, Marshall *et al.* 2001).

The turbid zone reveals a reduction of bacteria and less damage to the collagen matrix compared to infected dentine. Transparent dentine is sclerotic or harder than normal dentine, is hypermineralised due to occlusion of the tubules with mineral, and acts as a barrier to penetration of substances. (Kuboki *et al.* 1977), which makes this zone reparable by the dentine-pulp complex. The sub-transparent area can be considered as the transition layer between the transparent zone and normal dentine (Marshall *et al.* 2001).

Experimental evidence suggests that the bacteria in the biofilm are the main driver of the caries process (Kidd *et al.* 2008). It appears that simple removal of biofilm or sealing of bacteria within the cavity can arrest the progression of the lesion (Kidd and Fejerskov 2004).

2.2 Reactions of the dentine-pulp complex to dental caries

The dentine and the pulp should be considered as one vital organ (the dentine-pulp complex) because they are similar in development, structure and function (Ogawa *et al.* 1983, Orchardson and Cadden 2001). Pain and inflammation in the pulp are mainly attributed to the permeability of the dentine tubules that will conduct the by-products of metabolic activities of the carious lesion and possibly a few bacteria to the pulp (Heymann *et al.* 2014). It has been proposed that molecules from bacteria and their products and/or components of dentine's matrix breakdown can initiate a biological reaction in the pulp. In slowly progressing caries this can induce dentine regeneration, in contrast to more aggressive carious lesions, which will create more intense cellular and inflammatory response in the pulp (Cooper *et al.* 2010).

It has been noted that pulp inflammatory reactions occur while the caries process is still limited to the enamel at an early stage of the carious process (Brännström and Lind 1965). At an early stage of the carious lesion, the pulp reaction is activated through the spread of stimuli from the cariogenic biofilm on the enamel surface through the rod/interrod enamel, which leads to a reduction in the odontoblast-predentine region before the start of peritubular dentine mineralisation. Furthermore, reactions in the sub-odontoblastic layer involved fibroblast-like cells invading the cell-free zone (Bjørndal *et al.* 1998). In a more advanced stage, the cytoplasm: nucleus ratio of the odontoblast cells is reduced with a reduction in the predentine zone. There is more proliferation of fibroblast-like cells in the cell-free zone. When the lesion is close to EDJ, there will be an increase in the growth of predentine matrix with an increase in collagen type I synthesis in the predentine zone (Bjørndal *et al.* 1998).

During the stages of progression of the carious lesion, the dentine-pulp complex is able to develop defensive cellular reactions against external injuries (Stanley *et al.* 1983). The defence mechanisms of the vital dentine-pulp complex, in addition to cellular changes in the pulp, include a reduction in the dentine permeability because of the diffusion of plasma proteins into the dentinal tubules, many studies investigated this phenomenon *in vitro* (Pashley *et al.* 1982, Pashley *et al.* 1984, Pashley 1996, Hahn and Overton 1997). Also, defences of the dentino-pulpal complex include mineral deposition (sclerosis) in the dentinal tubules, which result in their gradual occlusion and decrease in dentine permeability, and reduce sensitivity to cold and rapid air movements (Fejerskov and Kidd 2008).

Sclerosis of the dentine is a natural aging phenomenon which results in gradual mineralisation of peritubular dentine and leads to complete occlusion of dentinal tubules, while in the case of caries, accelerated sclerosis results either from initial mineralisation of peritubular dentine followed by calcification of the odontoblastic process, or initial intra-cytoplasmic calcification followed by secondary peri-odontoblastic mineralisation (Frank and Voegel 1980). In slowly progressing or chronic carious lesions, the occlusion of tubules progresses not only as a result of peritubular dentine precipitation but also of intratubular dentinal precipitation. It has been found that in addition to intratubular hydroxyapatite crystals, there are large rhomboidal crystals (whitlockite) in the dentinal tubules (Fejerskov and Kidd 2008). Sclerotic dentine appears translucent under the light microscope due to a reduction in light scattering through the affected tissue, therefore, it is referred to as the translucent or transparent dentine zone (Fejerskov and Kidd 2008).

When the carious lesion comes in contact with the EDJ, there is a decrease in the size of odontoblasts with the loss of their columnar shape, with an increase in cellular activity in the cell-free zone along with deposition of reactionary dentine by affected odontoblastic cells (Couve 1986, Smith *et al.* 1995). Reparative/reactionary dentine is produced in response to caries, operative procedures and attrition to protect the pulp chamber (Stanley *et al.* 1983).

2.3 Overview of Dentinogenesis

Dentinogenesis is a multifaceted cellular process that is regulated by various factors. From a histological point of view, dentine can be classified as primary, secondary and tertiary (Kuttler 1959). Secondary dentine is produced after completion of tooth formation because of physiological conditions subsequent to the formation of primary dentine during tooth formation.

Tertiary dentin (tertiary dentinogenesis) can be divided into reactionary dentin and reparative dentin: reactionary dentin is a tertiary dentin formed by surviving post-mitotic odontoblast cells as a reaction to stimuli such as caries, while reparative dentin is formed by odontoblast-like cells (Smith *et al.* 1995). Reactionary dentinogenesis represents an up-regulation of the synthetic/secretory activity of the existing odontoblasts, whilst reparative dentinogenesis involves recruitment of stem or progenitor cells, induction of their differentiation into odontoblast-like cells and then, up-regulation of their synthetic/secretory activity. Thus, reparative dentinogenesis involves a more complex cascade of biological processes (Smith *et al.* 2005). The mechanisms that cause significant different histological appearance between primary and tertiary dentine are unknown. External stimuli may modify Odontoblast’s secretory function causing alterations in extracellular macromolecules elaborated during dentinogenesis (Moses *et al.* 2006). It is believed that slowly progressing caries or tooth wear may encourage reactionary dentine formation, while reparative dentine formation is induced in response to a more intense stimulus such as that caused by a rapidly progressive caries lesion (Smith and Lesot 2001).

Bioactive molecules and growth factors secreted by Odontoblasts are integrated into dentine matrix during primary dentinogenesis (Smith and Lesot 2001). Bacterial products such as acids during caries (Dung *et al.* 1995) and pulp capping agents application, such as calcium hydroxide and MTA, cause release of these bioactive molecules and growth factors (Tomson *et al.* 2007), which will drive a cascade signalling reaction of regeneration (Smith *et al.* 2012). Therefore, MTA induces repair of the pulp by solubilising the bioactive molecules from dentine, which can up-regulate proliferation and chemotaxis in the pulp (Tomson *et al.* 2016).

2.3.1 Reactionary dentinogenesis

The formation of reactionary dentine by odontoblasts begins as a result of the release of bioactive molecules from demineralised dentine matrix due to acidic by-products of the caries process; these molecules will initiate a cascade reaction that stimulates odontoblasts to secret modified atubular dentinal matrix with altered biochemical properties (Smith *et al.* 1995). These bioactive molecules mainly are transforming growth factors TGF-1, TGF-3 and bone morphogenic protein BMP-7 (Goldberg and Smith 2004). In addition, there are other non-phosphorylated, non-collagenous proteins such as dentine sialoprotein (DSP), dentine phosphoprotein (DPP), dentine matrix protein-1 (DMP-1) and osteopontin, which are able to regulate hydroxylapatite crystals formation (He *et al.* 2003). Transforming growth factor-β (TGF-β) and bone morphogenic protein (BMP) have significant roles in tooth development and tissue repair (Ruch *et al.* 1995, Sloan and Smith 1999, Tucker and Sharpe 1999).

An essential extra-cellular matrix protein for dentine formation is Dentine sialophosphoprotein (DSPP), which is secreted by odontoblasts as a multi-domain for dentine sialoprotein (DSP) and it helps mineral deposition (Yamakoshi *et al.* 2005, Yamakoshi *et al.* 2006). It has been found that there is increased amount of dentine sialoprotein (DSP) during reactionary dentinogenesis (Charadram *et al.* 2012). In carious teeth, gene expression levels of DSP in odontoblasts were higher than that in healthy teeth. Gene expression level was inversely related to the distance between odontoblast layer and carious lesion front. The amount of reactionary dentine deposition was related to the variation in gene expression (Farahani *et al.* 2010).

During healthy and pathological conditions, remodelling of connective tissue is intermediated significantly by the matrix metalloproteinase (MMP) (Nagase and Woessner 1999). MMP-2 has expressed abundantly in odontoblasts and dentine (Mazzoni *et al.* 2007). The source of MMP-2 is inactive pro-MMP-2 secreted by osteoblasts. Membrane type-1 matrix metalloproteinase (MT1-MMP) and tissue inhibitor of metalloproteinase-2 (TIMP-2) regulate activation of pro-MMP-2 into MMP-2 (Kurschat *et al.* 1999). MMP-2 cleaves DSPP into DSP (Yamakoshi *et al.* 2006).

During caries progression, lipopolysaccharides expressed by Gram-negative bacteria trigger the TLR-4 receptors. TLR-4 receptors are a toll-like receptor family possessed by odontoblasts which have the capacity to recognise pathogen-associated molecular patterns such as those present in lipoteichoic acid, a cell wall component of Gram-positive bacteria mostly present in the initial lesion and lipopolysaccharide, a cell wall component of Gram-negative bacteria present in more advanced lesions (Farges *et al.* 2009).

Triggering TLR-4 receptors activate MMP-2 by upregulation of TIMP-2 and MT1-MMP. Active MMP-2 cleaves DSPP into DSP. DSP deposits on newly synthesised collagen by odontoblasts and initiates hydroxyapatite crystallisation by forming nucleation sites. TGF-β release from dentine matrix because of bacterial acids or MMP-2, upregulate collagen synthesis by odontoblast (Fig. 2-3) (Charadram *et al.* 2012).

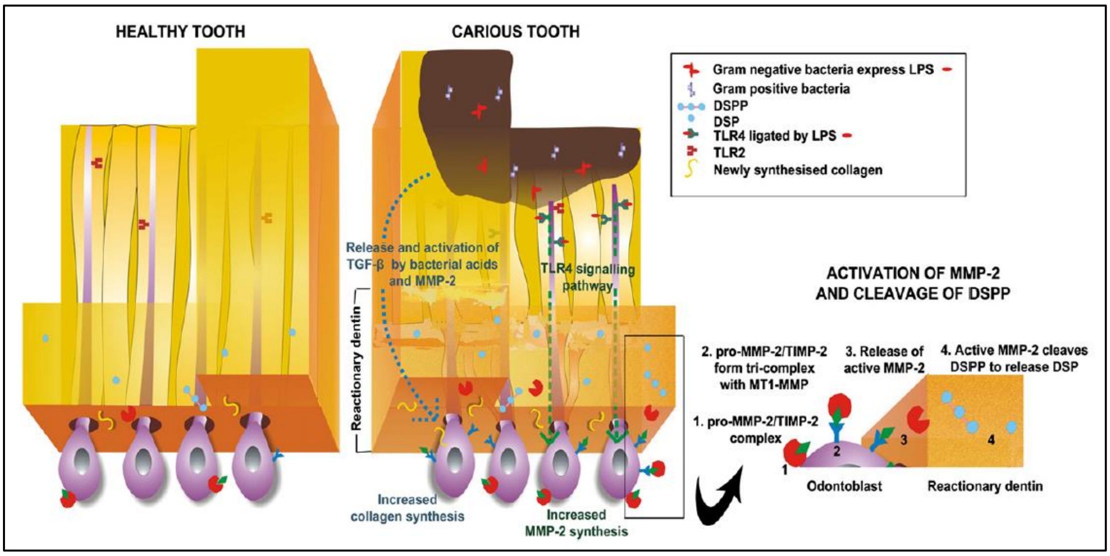


Figure 2-3: Diagram represents reactionary dentinogenesis process. source: (Charadram *et al.* 2012).

The inflammatory process accompanying dentinogenesis also starts when TLR-4 receptors in odontoblasts are activated by pathogen-associated molecules, which will lead to activation of the nucleic factor kappa B (NF-κB) signalling pathway which mediates cellular inflammatory reactions (Veerayutthwilai *et al.* 2007). Antimicrobial peptides, chemokines, and pro-inflammatory cytokines such as interleukin-1a, -1b (IL-1a, -b) and tumour necrosis factor-a (TNF-a) are released, resulting in upregulation of immune cells and further regulation of immune and inflammatory process in the dental pulp (Silva *et al.* 2004, Cooper *et al.* 2010).

Many defence and repair mechanisms in the pulp against caries may contribute to the destruction of the pulp itself. The inflammatory process may promote regenerative mechanisms, including angiogenesis. Defence and repair process in the pulp modulated by growth factors, neuropeptides, cytokines and chemokine released from pulp cells, immune cells and dentine matrix. The release of proteolytic enzymes and other degrading substances by immune cells in addition to MPPs during pulp defence cause degradation of DNA, lipids and proteins, leading to further cellular and tissue damage (Cooper *et al.* 2010).

Further to cellular inflammatory reactions of the pulp, there is accompanying vasodilatation of blood vessels to facilitate transport of immune and inflammatory cells into the site of disease (Cooper *et al.* 2010). Furthermore, neurogenic inflammation, which is a response of afferent nerves to bacterial antigens, will release neuropeptides which will further recruit and activate immune cells (Haug and Heyeraas 2006). Interestingly, it has been found that these neuropeptides have a role in the regulation of dentinogenesis in addition to their role in the development of dental pain (El Karim *et al.* 2009).

In conclusion, data support that the process of dentinogenesis, whether reactionary or reparative, usually occurs after resolution of the inflammatory process, which is a prerequisite for regeneration of the pulp (Goldberg *et al.* 2008), Pro-inflammatory cytokines in chronic inflammation may inhibit odontoblastic-like cell differentiation from stem progenitor cell populations, until inflammation is resolved. This leads to a balance between reparative process initiation and ensuring inflammation in the pulp evoked by the level of inflammatory mediators, where high levels lead to persistent inflammation and low levels can initiate the regenerative process (Cooper *et al.* 2010).

2.3.2 Reparative dentinogenesis

In the case of severe injury to the pulp, with or without exposure, the odontoblasts will be destroyed, which leads to differentiation of odontoblastic-like cells from underlying pulp cells in order to secrete a reparative dentine matrix (Tziafas 2004). Reparative dentine develops either as osteodentine, when the odontoblastic-like cells are entrapped in the reparative dentine, or orthodentine, which is tubular but without cells (Goldberg *et al.* 2003). The origin of these odontoblast-like cells may be derived from different cell populations in the pulp with no single progenitor for differentiation of these cells (Sloan and Smith 2007).

The cell-rich layer of Höhl adjacent to the odontoblasts is a possible site for these progenitor cells; these cells were proposed to be derived from the dividing pre-odontoblasts before their terminal differentiation into odontoblasts (Cotton 1968, Tziafas 1995). Other sites are from fibroblasts, undifferentiated mesenchymal cells, perivascular cells and post-natal dental pulp stem cells (Ruch *et al.* 1995, Gronthos *et al.* 2000).

Also, micro-vasculatures of the dental pulp are resided by stem cell-like cell populations with high proliferative potential and self-renewal capabilities (Gronthos *et al.* 2000, Shi and Gronthos 2003, Okiji and Yoshiba 2009). Differentiation of dental pulp stem cells into odontoblast-like cells and deposition of mineralised tissue is demonstrated *in vitro* (Liu *et al.* 2005).

Cell migration is facilitated by the expression of integrin subunits which occur when TGF-ß1 and BMP molecules in the solubilised dentine matrix come in contact with the pulp cells along with some pro-inflammatory cytokines (Mitsiadis and Rahiotis 2004). Activator protein-1 (AP-1) is a key regulator of cellular migration, proliferation, and differentiation and has been indicated in enhancing the expression of TGF-ß1, collagen type I, alkaline phosphatase, and osteocalcin (Smith and Lesot 2001). Notch, nestin, cadherins, and connexins also contribute to the initiation of odontoblast-like cell differentiation (Mitsiadis and Rahiotis 2004).

It is obvious that there are many differences in cell types included in tertiary, which could affect the resultant regenerative responses of the pulp. However other factors mediate pulpal inflammatory response such as strength and amount of adverse challenge which may result in dominant pulpal inflammatory response (Sloan and Smith 2007). The death of cells of the cell-rich zone of Höhl may occur simultaneously with the death of the affected odontoblasts because of the injury (Cotton 1968), Still, such cells are able to migrate from other unaffected areas of the pulp (Sloan and Smith 2007). Although pulpal inflammation is well known as a regeneration mediator, its persistence leads to regeneration inhibition (Rutherford and Gu 2000). Inflammation presence compromises recruitment and differentiation of stem cells during regeneration; however, it is uncertain whether this reflects a direct effect on the cells or on the molecular signalling processes responsible for their differentiation. (Sloan and Smith 2007).

2.4 Dentine-pulp complex response to restorative procedures

After cavity preparation in rat molars, Apoptosis of odontoblast occurs, this may indicate a greater impact of surgical phase on pulpal damage during restoration in comparison to restorative materials (Matsumoto *et al.* 2001). Different restorative factors have been investigated *in vitro* (Murray *et al.* 2000a) and the likelihood of pulp cell death underneath cavity preparations is well documented (About *et al.* 2001a). Postoperatively, the main reparative response of odontoblast to a cavity is the secretion of reactionary dentine. Also, it has been noted that there is an inverse relationship between remaining dentine thickness and pulp injury and repair (Murray *et al.* 2000b). This is very important, especially in the case of the acid etching procedure, which is important to increase the longevity of restorations. In teeth with remaining dentine thickness less than 300 μm, acid etching may lead to severe irritation to odontoblasts and persistent inflammation in the pulp due to high permeability caused by acid etching (Pashley *et al.* 1983) and fast penetration of acid into the pulp (Qvist *et al.* 1989).

Many studies found a severe inflammatory response with the aspiration of odontoblasts into dentinal tubules, along with secretion of reactionary dentine in teeth conditioned with the acid etchant in comparison to non-etched teeth (Qvist *et al.* 1989, Hebling *et al.* 1999, de Souza Costa *et al.* 2002). Reduction in odontoblasts numbers was found beneath etched dentine in comparison to non-etched dentine (Fujitani *et al.* 1992) as well. Also, it has been noted that a decrease in remaining dentine thickness from 1 mm to 0.25 mm in class V restorations can cause an increase in pulpal inflammation, even in the absence of bacteria. However, the presence of bacteria, regardless of the amount of remaining dentine thickness, was always associated with pulpal inflammation (About *et al.* 2001b).

In addition, data confirm that heat is one of the main causes of injury to pulp tissue during cavity preparation, drill rotation speed, cutting instruments' size and type, time of contact with dentine, pressure exerted on handpiece, and restoration material temperature all can play a significant role in raising temperature of the pulp (Zach 1972, Hatton *et al.* 1994, Ottl and Lauer 1998, Ohmoto *et al.* 1994, Murray *et al.* 2000a). It has been found that no use of coolant during cavity drilling may lead to odontoblast aspiration (displacement of odontoblasts into dentinal tubules) and inflammatory cells infiltration into the pulp (Kramer 1961). It has been found that coarse and ultra-coarse grit diamond burs can cause more rise in intra-pulpal temperature in comparison to fine grit diamond burs. The same happens with an increase in handpiece rotation speed and pressure, which cause intra-pulpal temperature rise (Ottl and Lauer 1998, Hatton *et al.* 1994).

In deep carious lesions, encouraging the dentine-pulp complex to respond to carious lesions by retaining caries-affected dentine also reduces the risk of unnecessary pulp exposure in deep cavities and excessive tissue destruction, and maximises the reparative potential of the dentine-pulp complex (Pugach *et al.* 2009). Complete dentine caries removal is not essential to stop caries progression, it has been found that carious lesions arrested by partial excavation of carious dentine and good sealing of the tooth cavity (Oliveira *et al.* 2006). The position of the tooth/root in the jaw should also be taken into consideration in the interpretation of pulpal reactions, as this will affect the vascularisation of the tooth and subsequently the healing potential of the dentine-pulp complex to injuries (Qvist *et al.* 1989).

Chemo-mechanical caries removal systems such as Carisolv™ gel have been introduced to facilitate selective removal of irreversibly damaged dentine and maintain reversibly affected dentine, which can decrease pulp exposure potential, especially in teeth with deep carious teeth (Banerjee *et al.* 2000b). The effect of Carisolv™ gel on pulp tissue has been compared to normal saline as a control. After exposure for 10 minutes, pulpal tissue exhibited a slight inflammatory response to both test materials after one day; however, there was localised haemorrhage in the control group; after one month, inflammation subsided in both groups with structurally integral pulp tissue (Bulut *et al.* 2004). This is in agreement with other studies, which concluded that Carisolv™ gel can have a bactericidal effect on pulp tissue with the ability to stimulate reparative dentine formation in the case of pulp exposure, and have minimal adverse effect on the pulp (Dammaschke *et al.* 2001, Young and Bongenhielm 2001). However, in dentine, Carisolv™ gel causes the destruction of odontoblastic processes and maintains healthy collagen fibrils (Dammaschke *et al.* 2002). In animal studies, Carisolv™ gel did not cause an adverse pulpal reaction in healthy rat teeth and had a similar effect to calcium hydroxide paste (Dammaschke *et al.* 2006).

2.5 Dentine-pulp complex response to restorative materials

One of the objectives of applying restorative materials in restorative dentistry is to maintain the health of the pulp in addition to restoring the function and aesthetic of teeth. The chemical activity of restorative materials, as well as mechanical trauma from the restorative procedures, can stimulate an inflammatory reaction in the pulp (Okiji *et al.* 1997). Although low-level pulpal inflammation can be caused by most restorative materials when used on young permeable dentine, higher inflammatory response is expected if there was associated bacterial leakage, which is a factor that should be taken into consideration in case of evaluation of pulp adverse effects associated with restorative materials (Murray *et al.* 2002c, Qvist *et al.* 1989). Although restorative materials have a mild effect on the pulp, still the pulp will react to noxious stimuli from restorative materials with an inflammatory reaction (Hilton 2009). Data regarding materials with adverse effect on pulp of teeth, such as amalgam, resin-based materials, liners and bases suggest that material-related sequelae can either be cytotoxic by destroying the pulp cells, or immunosuppressive by reducing the ability of the pulp to respond to a bacterial invasion (Hilton 2009), However, many components of restorative materials have a beneficial or enhancing effect on the dentine-pulpal complex, for example, eugenol which can diffuse into dentine and pulp from a zinc oxide eugenol base or cement to inhibit bacterial metabolism and reduce pulpal inflammation and pain (Hume 1988). Similarly, fluoride diffuses from fluoride-containing restorative materials into adjacent enamel, dentine and cementum, leading to remineralisation (Forss 1993).

Ions such as silver, copper and mercury, which are released from amalgam, may have an adverse effect on the pulpal tissue by diffusion through dentinal tubules (Wataha *et al.* 1994). Amalgams that contain zinc have higher cytotoxicity compared to other amalgams, and high copper and low copper amalgams showed similar cytotoxicity (Kaga *et al.* 1988). Zinc is considered as the major cause of amalgam’s cytotoxicity (Espevik *et al.* 1982). Because of the unreacted mercury, freshly mixed amalgam is more cytotoxic than set amalgam (Tronstad and Wennberg 1980). Mercury compounds exhibited higher cytotoxicity compared to the resin composite constituents because of the ability of the mercury compounds to interfere with the cellular metabolism and function leading to cell swelling and finally cell death (Reichl *et al.* 2006).

Resin composites and bonding resin can also cause marked inflammation, damage to the pulp and dilatation and congestion of blood vessels when placed in deep dentine, even in the absence of bacterial microleakage in both animal and human teeth *in vivo* (Hörsted *et al.* 1987, Qvist *et al.* 1989, Cvek *et al.* 1990, Fujitani *et al.* 1992, Modena *et al.* 2009). In fact, compounds such as triethylene glycol dimethacrylate (TEGDMA) and camphoroquinone, 2-hydroxyethyl methacrylate (HEMA) components, which diffuse from bonding resins and resin composites through dentine into the pulpal tissue in small quantities (micrograms) within hours and days after placement, can cause adverse cytotoxic effects on the pulp (Hume and Gerzina 1996). During direct pulp-capping with adhesive resins and incomplete polymerisation of adhesive resin monomers may lead to leaching of un-polymerised monomers into the pulpal tissue, causing severe inflammation and a cytotoxic effect (Pashley 1988, Modena *et al.* 2009). The release of unreacted monomer (1.5-5% of the methacrylic group remain unreacted) is because of mechanical, thermal and chemical factors that inhibit complete polymerisation, which is enough to initiate a cytotoxic effect (Stanislawski *et al.* 1999). It has been concluded that a persistent inflammatory reaction, dentine bridge formation failure and pulpal pathosis were associated with bonding agents when used as pulp-capping agents, therefore the use of acidic agents and adhesive resins as pulp-capping agents is contraindicated (de Souza Costa *et al.* 2000).

Studies by Costa and his colleagues found that the pulpal inflammation and damage was related to the amount of remaining dentine thickness between the cavity floor and the pulpal tissue, and also to the etching procedure in dentine. Resin tag penetration increased in etched dentine samples, which was associated with moderate inflammatory response and pulpal tissue disorganisation in comparison with un-etched dentine or dentine coated with calcium hydroxide before etching; moreover, they found that the presence of bacteria was not correlated with pulpal inflammation (de Souza Costa *et al.* 2002, de Souza Costa *et al.* 2006). Similar results were obtained when deep cavities were restored with resin composite after etching and bonding of dentine in the presence or absence of bacterial contamination, where the inflammation was evident in the pulp of these cavities in comparison with the pulps of calcium hydroxide-lined teeth (Hebling *et al.* 1999). Calcium hydroxide's antibacterial effect probably leads to a reduction of the bacterial insult on the pulp of these teeth.

Glass ionomer cement (GIC) was introduced 45 years ago (Wilson and Kent 1971). GICs generally are described as acid-base reaction cements and they are derived from organic acids (aqueous polymeric acid) and a glass component (fluoro aluminosilicate) (Sidhu 2011). Studies conducted on human exposed pulps to assess the biocompatibility of GIC showed increased inflammatory cell infiltration and more odontoblast aspiration compared with controls when GIC were placed in deep non-exposed teeth (Tobias *et al.*

1978, Cooper 1980, Plant *et al.* 1984). Moreover, a study by Müller and his colleagues found similar results and recommend that GIC is not suitable for direct pulp capping (Müller *et al.* 1990).

On the contrary, when GIC Fuji IX was used as indirect pulp capping agent in deep unexposed teeth, although there was higher inflammatory response compared to that of the control unfilled cavities after eight days, there was complete recovery of pulpal inflammation after one month associated with reparative dentine formation (Six *et al.* 2000). In conclusion, GIC is a suitable material as an indirect pulp-capping agent and dentine substitute with acceptable biocompatibility in deep unexposed cavities (Sidhu 2011)

In the late 1980s, resin-modified glass ionomer cement (RMGIC) was introduced (Mitra 1989). To improve the mechanical properties of GIC, photo-initiators and hydrophilic monomers were added (Cattani-Lorente *et al.* 1994). Many *in vitro* studies showed higher cytotoxicity of RMGIC in comparison to conventional GICs (McCabe 1998, de Souza Costa *et al.* 2003). Application of RMGIC as a pulp-capping agent in humans resulted in a moderate to intense inflammatory response with a large necrotic zone. Congested venules associated with extravasation and neutrophilic infiltration were observed in comparison to teeth capped with calcium hydroxide, which showed slight to moderate inflammatory response with a large zone of coagulation necrosis, therefore pulp-capping with RMGIC is contraindicated (do Nascimento *et al.* 2000). This may be attributed to the cytotoxic effect of leachable HEMA similar to that observed in adhesive resins (Hashimoto *et al.* 2004). It has been noted that the aqueous extract of RMGIC obtained at 24h was less toxic than 48h and 72h on odontoblasts cell lines (time-dependent). Implantation of RMGIC subcutaneously resulted in low to high inflammatory response, which resolved over time in rats (Souza *et al.* 2006).

Zinc oxide eugenol (ZOE) is used widely in dentistry as a cement, base and liner, or as a temporary filling (Hilton 2009). Clinical investigation on human subjects showed that ZOE causes chronic inflammation in the pulp, no healing and no dentine bridge formation when placed directly on the pulp, in comparison calcium hydroxide after a 12-week observation period (Glass and Zander 1949). The thin dentine barrier between the pulp and ZOE can exaggerate the pulpal response to leachable eugenol, which may cause pulp hyperaemia, inflammatory cell aggregation, reduction in the number of odontoblasts, and the presence of cell nuclei in the tubules (Bra and Nyborg 1976). Thick dentine sections provided better protection to the pulp than thinner sections, and this has been suggested to be due to the presence of calcium in the dentine tubules, which has a chelating effect on the eugenol, limiting its ability to diffuse (Markowitz *et al.* 1992). Avoiding direct contact with the pulp with sufficient intact dentine to allow the eugenol's analgesic and anti-inflammatory effect to predominate over its toxic potential will provide the maximum benefit from ZOE (Markowitz *et al.* 1992).

2.6 Diagnosis of pulpitis

Correct diagnosis of pulpal status/disease is a very important step in choosing suitable treatment and subsequently predicting prognosis. However, it is very difficult to describe the pulp inflammatory status clinically in patients, because of the lack of correlation between pulpal histology and clinical symptoms (Simon *et al.* 2012, Dummer *et al.* 1980, Baume 1970). Clinical findings, symptoms presentation and history are the elements of clinical diagnosis (Sigurdsson 2003). Unfortunately, there have been many attempts made in the past to classify pulpal conditions and to introduce clinical tests that can help to reach a correct clinical diagnosis (Dummer *et al.* 1980).

However, findings of Dummer et al. (1980) were similar to those of Seltzer *et al*. (Seltzer *et al.* 1963), where it was difficult to differentiate clearly between saveable (i.e. pulpal inflammation with no necrosis) and non-saveable (i.e. pulpal inflammation with necrosis) teeth, depending on signs and symptoms; therefore a final diagnosis can be made only after histological examination of the tooth, which is clinically impractical (Dummer *et al.* 1980). However, recently it has been found that there is a good correlation between the defined criteria used in the clinical diagnosis of reversible/irreversible pulpitis and the histological findings. There was high agreement between the clinical and histological findings especially in cases with reversible pulpitis (96.6%), compared with irreversible pulpitis cases which showed that 15% of them were histologically identified as reversible pulpitis. However; there is a need to improve means of pulp diagnosis (Ricucci *et al.* 2014).

Clinically, pulpal status can be classified into healthy pulp, reversible pulpitis, irreversible pulpitis and necrotic pulp, in addition to other periapical status categories (Sigurdsson 2003). The most recent classification was suggested by the American Board of Endodontics in an attempt to standardise the classifications and establish consistency in terms.

The following table displays the clinical classification of pulp and periapical disease produced by the American Board of Endodontics (Glickman 2009) (Table 2-1).

Table 2-1: Clinical classification of pulp and periapical disease (AAE, 2009).

Pulp Disease	**History**	**Clinical Examination**	**Radiographic Examination**
Normal pulp “Pulp is vital with no inflammation”	Symptom free	“Normal” response to thermal cold testing as compared to healthy control teeth. The tooth may not be histologically normal.	Normal radiographic findings.
Reversible pulpitis “Pulp is vital, any inflammation should resolve and the pulp return back to normal following appropriate management of the aetiology”	Short, sharp pain stimulated by hot, cold or sweet stimuli. Few seconds duration and disappears when the stimulus is removed. Negative history of spontaneous pain.	Aetiology: exposed dentine, caries and deep restorations. No pulp exposure, sinus, fistula, swelling of periodontal tissues, abnormal tooth mobility, spontaneous pain or sensitivity to percussion. Normal appearance of adjacent gingiva and normal tooth colour. Pulp sensibility tests elicit an exaggerated response which disappears when the stimulus is removed.	No radiographic changes in the periapical region.
Irreversible pulpitis (Symptomatic/asymptomatic) “Vital inflamed pulp is incapable of healing and root canal treatment is indicated”	If symptomatic: Dull, throbbing pain, may be acutely sharp. Usually spontaneous but may be exacerbated upon thermal stimulus and lingers for minutes/hours. Pain may be accentuated by postural changes such as lying down or bending over. Analgesics are not effective.	Aetiology: Deep caries, extensive restorations, or fractures exposing the pulp tissues. May or may not exhibit pulp exposure, sinus and fistula, swelling of periodontal tissues, abnormal tooth mobility, spontaneous pain or sensitivity to percussion. Pulp sensibility tests elicit an exaggerated response which lingers when the stimulus is removed.	May or may not display radiographic changes in the periapical region such as PDL widening or lesion.

Pulp Disease	**History**	**Clinical Examination**	**Radiographic Examination**
Pulp necrosis “Partial or complete death of the dental pulp, necessitating root canal treatment”	No symptoms. May or may not be preceded by symptoms of irreversible pulpitis.	No response to pulp sensibility testing. May or may not exhibit pulp exposure, sinus, fistula, swelling of periodontal tissues, abnormal tooth mobility, and sensitivity to percussion.	May or may not display radiographic changes in the periapical region such as PDL widening or lesion.
Periapical Disease	**History**	**Clinical Examination**	**Radiographic Examination**
Normal apical Tissues		No sensitivity to percussion as compared to a control tooth.	Lamina dura surrounding the root is intact and the periodontal ligament space is uniform.
Symptomatic apical periodontitis “Represents inflammation of the apical periodontium”	Pain on biting.	Sensitivity to percussion.	May or may not be accompanied by radiographic changes.
Asymptomatic apical periodontitis “Inflammation and destruction of the apical periodontium that is of pulpal origin”	No symptoms	No sensitivity to percussion.	Periapical radiolucency
Acute apical abscess “Inflammatory reaction to pulpal infection and necrosis”	Rapid onset, spontaneous pain, malaise, fever and lymphadenopathy	Extreme sensitivity to percussion, varying degree of mobility, pus formation and swelling of associated tissues.	There may be no radiographic signs of destruction. But there may be a loss of lamina dura and widening of the PDL.

Chronic apical abscess “Inflammatory reaction to pulpal infection and necrosis”	Gradual onset of pain, little or no discomfort.	Not sensitive to percussion but may “feel different”. Intermittent discharge of pus through an associated sinus tract.	Periapical radiolucency

2.6.1 Pain history

Conclusive diagnosis of the cause of the pain should be preceded by registering associated pain history, signs and symptoms alongside the clinical examination of the patient (Sigurdsson 2003). Pulp pain is conducted by two types of nerve fibres in the pulp: the fast conducting myelinated A-delta nerve fibres, which are located in the coronal portion of pulp, mostly in pulp horns; and slow-conducting unmyelinated C-fibres, which are located in pulp proper. The A-delta fibres have a fast conduction velocity of 12 to 30 m/s. The slow C-fibres have a conduction velocity of 0.5 to 2 m/s (Bender 2000a). Pain associated with electrical stimulation of A-delta fibres is characterised by a pricking nature (i.e. sharp pin sticking), while that of C-fibres is characterised by an aching type of pain. C-fibres require stronger electric current (37.4 μA) than standard electrical pulp tester current, which is used in stimulating A-delta fibres (9.9 μA) (Narhi *et al.* 1979).

An effective predictor of pulpal inflammation reversibility is the patient's report of pain intensity and feature, with results indicating that the McGill Pain Questionnaire, when used alone, correctly predicted diagnosis and treatment outcome in 73% of patients (Grushka and Sessle 1984). It was noticed that frequency of pain increases simultaneously with histopathological adverse manifestations. In a study by Bender *et al.* (2000), the majority (80%) of patients manifested chronic partial pulpitis with partial necrosis (irreversible damage) histopathologically when they reported a previous history of pain compared to only 20% who showed no necrosis with mild inflammatory response (reversible damage) in histopathological examination. Accordingly in a clinical setting, determining the histopathosis of a painful pulpitis can be achieved by registering the previous history of pain from the patient which can help in addition to the clinical examination in reaching a conclusive diagnosis of the reversibility of the painful pulpitis (Bender 2000a).

2.6.2 Clinical diagnostic tests

Clinical diagnostic tests used to diagnose pulpal status were introduced in the 1890s when Marshall used electric current to assess pulp vitality and Walkoff used thermal stimuli and radiography (Walkhoff 1898, Bender 2000a). Pulp testing strategies involve pulp sensibility testing such as thermal or electric pulp testing (EPT), which are based on activating the neural pathway of the pulp, initiating a sensory response. This most commonly undergoes by applying a thermal or electrical stimulus to the outer surface of the tooth to provoke a sensory reaction from A-delta nerve fibres which presents in the dentine-pulp complex leading to sharp and short sensation from the tested tooth (Gopikrishna *et al.* 2009). In the case of thermal stimulation, the nerve endings will act as mechanoreceptors for rapid fluid movements in dentinal tubules which will excite A-delta nerves and cause pain sensation. This cannot be achieved if there was a slow temperature change because of the slow fluid movement which will eventually excite thermoreceptors of C-fibres causing pain (Trowbridge *et al.* 1980). While EPT induces pain by introducing an electric stimulus that causes ions movement at the nerve endings and provoking rapid bouncing action in the nodes of Ranvier in myelinated nerve fibres (Bender 2000a).

Agents used for cold thermal tests are CO_2 snow, dichlorodifluoromethane, ethyl chloride and ice. Agents used for heat thermal tests are hot gutta-percha, impression compound and water. It has been found that there is no detrimental effect of a cold test on subjected teeth in terms of crack propagation or pulpal health (Rickoff *et al.* 1988) in contrast to a heat test, which may cause irreversible damage (Sigurdsson 2003).

In a study by Fuss and colleagues, it was found that ethyl chloride and ice are less reliable than EPT, CO_2 snow and dichlorodifluoromethane in producing a positive response in young, intact human premolar teeth, but EPT was less reliable than CO_2 snow and dichlorodifluoromethane (Fuss *et al.* 1986), However, another study in pulpless (teeth with root canal fillings) and pulpal diseased teeth (teeth with confirmed associated apical radiolucencies) found that there was no significant difference between electric and cold pulp tests in false positives however in multirooted teeth, there was false positive reading with cold test because of remnant of vital tissue in at least one canal (Peters *et al.* 1994). The sensitivities of cold, heat and electrical tests were 0.88, 0.86 and 0.76, respectively. Their specificities were 1 for all 3 tests. The negative predictive value was 0.90 for the cold test, 0.89 for the heat test, and 0.83 for the electrical test, and the positive predictive value was 1.0 for all 3 tests. The highest accuracy (0.94) and reproducibility (0.88) were observed for the cold test (Villa-Chávez *et al.* 2013).

Lack of reproducibility represents another shortcoming of the tests used for pulp sensibility; a study found that CO_2 and EPT were less repeatable and less time-consuming than laser Doppler flowmetry (laser Doppler flowmetry is a non-invasive, unbiased and consistent method for pulpal blood flow measurement). Although cold test with Endofrost was reliable, it was not accurate as well as EPT and CO_2; also it was less repeatable than ice and laser Doppler flowmetry. Ice (not CO_2) was the most repeatable but the least accurate and least reliable test (Chen and Abbott 2011). In general, both electrical and thermal tests have no ability to diagnostically differentiate between reversible and irreversible inflammation of the pulp (Sigurdsson 2003).

Although a positive subjective response from the patient to thermal stimuli or EPT designates operational nerve fibres, it is not a true indicator of the tooth vitality because it does not reflect the status of blood flow which is the true indicator of tooth vitality (Munshi *et al.* 2003). Teeth with lost sensory function due to trauma are not responsive to a pulp sensibility test, however they have intact vasculature (Yanpiset *et al.* 2001). Therefore, laser Doppler flowmetry and pulse oximetry, which assess the blood circulation of the pulp, are 'more accurate and objective methods for the assessment of pulp vitality (Hargreaves and Berman 2015).

In 1986, Gazelius and his colleagues used laser Doppler flowmetry in assessing blood flow in the pulp of vital teeth and where the output signal from the flowmeter was clearly distinguishable from that of non-vital teeth with good reproducibility (Gazelius *et al.* 1986). Laser Doppler flowmetry is a non-invasive, objective, painless, semi-quantitative and reproducible method, which has been proved to be a consistent method for pulpal blood flow measurement; therefore, it can be considered the gold standard in determining pulpal blood flow (Olgart *et al.* 1988, Gazelius *et al.* 1986). However, it has been noted that there are positive blood flow records from non-vital teeth because of blood flow in non-pulpal tissue which may be reflected as a signal recorded from the enamel surface (Hartmann *et al.* 1996). Therefore, when measuring pulpal blood flow, crown isolation is necessary to prevent false readings (Hartmann *et al.* 1996, Jafarzadeh 2009), however, it is difficult to eradicate the signal from periodontal tissues totally (Jafarzadeh 2009).

In 1991, the pulse oximeter was introduced in the field of pulpal pathology diagnosis. This is a non-invasive device indicating tooth vitality by measuring oxygen levels in pulpal blood vessels using multiple-wavelength optical plethysmography which determines the pulp oxygenation level of a tooth and the vitality of a tooth (Schmitt *et al.* 1991). A study found higher sensitivity and specificity of the pulse oximeter (1.0 and 0.95 respectively) compared to cold and electric tests (sensitivity 0.81 and 0.71

respectively, specificity 0.92 both), reflecting higher objectivity of pulse oximeter dental probe in determining tooth vitality (Gopikrishna *et al.* 2009).

Dual wavelength spectrophotometry technique showed a significant and reproducible ability to differentiate between air-filled, fixed tissue and oxygenated blood pulp spaces *in vitro* (Nissan *et al.* 1992). The instrument might be promising in determining the inflammatory status in the pulp, as it is able to determine pulp necrosis (Sigurdsson 2003).

Other types of tests such as percussion and palpation tests are used in combination with pulp sensibility tests in pulpal diagnosis. Although percussion and palpation elicited pains are more indicative of periapical inflammation, it has been found that they may have been associated with partial or total necrosis of the pulp, providing an indirect method to assess the pulp status to conclude irreversibility of the pulpal inflammation (Seltzer and Bender 1985). Another study found that percussion and palpation tests were effective in differentially diagnosing between pulpal and periapical conditions (Iqbal *et al.* 2007).

2.6.3 Radiographical diagnostic modalities

The findings of radiographical examination should always be evaluated together with the findings of other tests; radiographs cannot detect pulpal status directly but can explore the presence of caries and defective fillings, in addition to periapical radiolucency of endodontic origin which is indicative of partial or total necrosis in the pulp (Sigurdsson 2003). The ability to detect these changes is critical in determining the accurate diagnosis, treatment plan and assessment of outcomes. However, there are extensive variations in radiographical interpretations among different observers (Kaffe and Gratt 1988). In general, it was found that the most consistent radiographic features to identify healthy teeth are density, size and pattern of bone trabeculae; however, for non-vital teeth diagnosis, PDL width and lamina dura continuity were found to be reliable radiographic characteristics (Kaffe and Gratt 1988). Because of mineral loss, a lesion in lamina dura may yield a radiographic finding more easily than in cancellous bone (Barbat and Messer 1998). Also, lamina dura discontinuity has been considered as a sign of local or systemic disease, e.g. Paget's disease (Marks and Dunkelberger 1980)

However, due to dimensional limitations in conventional radiographs, which are two-dimensional images of three-dimensional structures, radiography is not a perfect diagnostic tool, in addition to the fact that radiographical changes may not reflect specific clinical and pathological features, therefore lesion presence may not be directly obvious, as well as its relationship to important anatomical structures (Huumonen and Ørstavik 2002). Other conditions, such as apical morphologic dissimilarities, adjacent bone thickness, x-ray angulations, and radiographic disparity, also influence radiographic interpretation (Halse *et al.* 2002). A study found that an artificial lesion in a certain location might and might not appear in the radiograph, depending on the type of the bone and the thickness of the cortical plate in that region (Huumonen and Ørstavik 2002). Other studies have shown that lesions confined to the cancellous bone could not be detected using standard periapical radiographs until the cortical plate is partially corroded (Marmary *et al.* 1999, Bender and Seltzer 2003a, Bender and Seltzer 2003b). Studies suggest that bone mineral loss should reach 30-50% in the periapical radiolucency to be visible radiographically (Bender and Seltzer 2003a, Bender and Seltzer 2003b). On the contrary, others found that cortical involvement is not necessary to visualise radiographically an artificial periapical lesion which is confined in the cancellous bone (Ford 1984, LEE and Messer 1986).

Several advanced radiographic techniques have been used in dentistry to detect bone lesions, such as densitometry, magnetic resonance imaging and ultrasound, cone-beam computed tomography (CBCT) and digital radiography (Huumonen and Ørstavik 2002, Cotti and Campisi 2004, Nielsen *et al.* 1995). Computed tomography (CT) provides three-dimensional information of teeth and jaws in comparison to periapical intraoral radiographs, first described to be used in endodontics in the 1990s (Tachibana and Matsumoto 1990, Marmary *et al.* 1999). However, its expensiveness, high radiation dose and dentist's office unavailability led to the introduction of CBCT (Lofthag-Hansen *et al.* 2007), which shows a limited field of the jaw similar to that of conventional periapical radiograph (Arai *et al.* 2001).). It was found that CBCT can provide relevant information in the diagnosis of periapical lesions in comparison to periapical radiographs because it overcomes the two-dimensional limitation, distortion and anatomical noise associated with periapical radiographs (Estrela *et al.* 2008, Patel *et al.* 2009, S. Patel *et al.* 2012). Furthermore, a recent study showed that CBCT was significantly more effective in detecting PA radiolucencies compared with periapical radiographs in patients with signs and symptoms of reversible pulpitis (Hashem *et al.* 2015).

A single CBCT 360-degree scan reconstructs volume images of projection data from a 50×40 mm, or 50×50 mm cylinders. Tomographic slices with a resolution from 0.125-2.0 mm can be presented in three perpendicular planes. With this volume data, it is possible to observe each part of the tooth (i.e. root) in three-dimensional planes by making new slices in the desired directions (Iwai *et al.* 2000). The axial, sagittal and coronal planes of the three-dimensional image of CBCT can identify anatomical complexities, periapical lesions and operational errors which may not be seen with conventional radiography due to the 2D image. Geometric distortion is a problem associated with conventional intraoral radiographs and CT scans where the voxels are anisotropic, in contrast to CBCT, where the voxels are isotropic, ensuring that the images produced are geometrically accurate and free from distortion (Patel *et al.* 2009).

For endodontics purposes, only a limited field of view scans is suitable as it will expose a limited field of the jaw only to the area of interest (Brown *et al.* 2014), This will yield a higher spatial resolution image in comparison to large field of view scans, and it will also decrease the dose to the patient to the level of “as low as reasonably achievable”, which should be considered in all cases as stated by a European Society of Endodontology position statement regarding the use of CBCT in endodontics (Patel *et al.* 2014).

2.7 Indirect pulp capping

Controlling caries and accompanying histopathological pulpal manifestations in deep carious lesions are challenging in operative dentistry (Mickenautsch *et al.* 2010). Studies found that the selective removal of the heavily infected dentine biomass while retaining affected dentine, shows favourable results in terms of tooth vitality preservation (Maltz *et al.* 2007, Gruythuysen *et al.* 2010, Orhan *et al.* 2010). To achieve indirect pulp protection, carious dentine near the pulp is preserved in order to avoid pulp exposure and is covered with a suitable material; this technique was described in 1967 (Kerkhove *et al.* 1967). However deep carious lesions represent a challenge to the dental practitioner in general, as there is no precise relation between the clinical manifestations of the disease and the histological changes in the dental pulp, which can affect the diagnosis and the subsequent care plan (Jespersen *et al.* 2014). Many dental practitioners (about 83%) favour complete removal of caries and jeopardise exposure of the pulp rather than leaving a layer of caries, and utilise indirect pulp-capping to restore the tooth (Oen *et al.* 2006). Pulp exposure after complete caries removal is one of the most common complications associated with the management of deep carious lesions in asymptomatic teeth (Magnusson and Sundell 1977, Ricketts *et al.* 2006), and because of the complexity and high cost of the root canal treatment and associated coronal restorations that will leave no choice to the patients other than extraction (Browning *et al.* 2013). Previous investigations have shown that conservative treatments of the exposed pulp resulted in a poor prognosis in follow-up trials (Barthel *et al.* 2000). Treatment of pulpal exposed teeth compared to non-exposed teeth is more challenging in terms of haemorrhage control, identifying and removing infected tissue and loss of dentine barrier, which can maximise adverse effects of capping materials on the pulp (Murray *et al.* 2002a)

Observational data about indirect pulp-capping studies and systematic reviews suggest that indirect pulp-capping is a successful pulp therapy alternative for treatment of deep carious lesions in both primary and permanent teeth with clinical success, and it is able to prevent further progression of carious lesion, regardless of the type of the capping materials used (Al-Zayer *et al.* 2003, Miyashita *et al.* 2007, Bjørndal 2008, Marchi JJ *et al.* 2009, Gruythuysen *et al.* 2010, Hashem *et al.* 2015). Indirect pulp-capping is performed with two approaches; either by incomplete caries removal with no subsequent re-entry (one visit) or stepwise caries removal with subsequent re-entry (two visits). Both techniques resulted in fewer numbers of pulp exposure (6% and 8% respectively) compared to 22% with the complete caries excavation technique in primary and permanent young molars (Orhan *et al.* 2010). Bjørndal *et al*. also reported a pulp exposure rate of 17.5% in stepwise excavation compared to 28.9% in complete caries excavation (Bjørndal *et al.* 2010).

2.8 Clinical approaches of indirect pulp capping

2.8.1 Incomplete caries removal with direct seal (no re-entry) (one visit)

Incomplete caries removal reduces significantly the risk of pulp exposure and post-operative pulp symptoms (Schwendicke *et al.* 2013a). A 77% reduction in risk of pulp exposure with partial caries removal was found compared to complete caries removal (Ricketts *et al.* 2013). Although concerns have been raised regarding lower fracture strength associated with partially excavated teeth compared to completely excavated teeth (Hevinga *et al.* 2010), the annual failure rate for teeth with incomplete caries removal was similar to that with completely excavated teeth, which have most failures due to pulpal reasons (pulpitis and abscess formation) (Schwendicke *et al.* 2013b).

After a three-year period, no re-entry carious dentine removal (single visit partial caries removal) have higher pulpal survival (91%) compared to stepwise excavation (re-entry) approach (69%) (Maltz *et al.* 2012). Currently, evidence suggests that there are no more complications associated with incomplete caries removal compared to complete caries removal (Maltz and Alves 2013). Many studies observed arrest of carious lesions size increase with low microbial loads after 6-19 months sealing period from oral cavity with a high success rate reaching 90.3%, regardless of the type of the material used (Bjørndal *et al.* 1997, Petrou *et al.* 2014). The protective effect is more significant when carious tissue is sealed definitively beneath restorations (incomplete caries removal with direct seal) than it is when the remaining carious dentine is removed in the second visit of stepwise excavation (Maltz and Alves 2013).

The review of teeth with deep caries lesions treated by incomplete caries removal over a 10-year period resulted in pulp survival rates of 97%, 90%, 82% and 63% at 1.5, 3, 5 and 10 years follow-ups, respectively (Maltz *et al.* 2011). Therefore, caries process control and caries arrest can be achieved by incomplete removal of carious dentine with good cavity sealing, complete removal of caries dentine lesion is not important in the control of the caries process (Maltz *et al.* 2002, Van Thompson *et al.* 2008).

Microbiologically, although there were higher microbial loads after partial caries removal compared to complete caries removal, there were similar microbial levels after sealing period in both groups, this suggests that there is no necessity to re-open the cavities after partial caries removal due to the persistence of bacteria (Lula *et al.* 2009). It will be satisfactory to alter the ecological and metabolic equilibrium in microbial community in caries to arrest caries process and enhance remineralisation as an alternative of making an attempt to eliminate all bacteria in the lesion (Bjørndal and Kidd 2005). In support for this approach, remineralisation of remaining carious dentine has been detected radiographically before (Maltz *et al.* 2002, Maltz *et al.* 2007).

The extent of caries removal (partial versus complete), still represents a challenge for studies investigating these approaches in term of how much caries tissue has been excavated or left. Furthermore, it is challenging to evaluate whether leaving more carious dentine will be associated with risks of failure and caries progression or advantages of fewer pulp exposures and symptoms expected from partial caries removal approach (Schwendicke *et al.* 2013a). Therefore, it is essential to evaluate caries excavation methods that can preserve tooth structure alongside efficiency in removing the active biomass of carious lesion to prevent progression of the lesion under tight coronal seal.

2.8.2 'Stepwise' excavation (re-entry)

It consists of incomplete excavation of carious dentinal lesion in proximity to the pulp followed by sealing of the cavity (Kidd 2004) to promote remineralisation of remaining carious dentine, decrease dentinal permeability, induce sclerosis and tertiary dentine formation and stop lesion progression (Massler 1978, Bjørndal 2001). After a period (8-24 weeks) has elapsed, removal of the remaining carious dentine has been suggested by re-entry to the cavity (Sowden 1956, Law and Lewis 1961, Eidelman *et al.* 1965, Magnusson and Sundell 1977).

Recent studies focused on exploring clinical, microbiological and radiographical outcomes of indirect pulp-capping with stepwise excavation. It has been found that cultivable microflora of remaining carious dentine change dramatically in numbers and species types after 4-6 months of treatment, confirming arresting of carious lesion progression clinically (Bjørndal and Larsen 2000), Also, removing central carious mass appears to be essential for control of caries progression (Bjørndal 2011). Furthermore, re-entry to the cavity has been suggested to enable researcher to assess persistent microbial phenotypes in excavated lesions (Bjørndal *et al.* 1997). The clinical appearance of reopened demineralised dentine was similar to that of the slowly progressing lesion with microbial flora similar to that of the slowly progressing lesion in root caries (Bjørndal and Larsen 2000).

In addition, a systematic review of the materials used for stepwise excavation concluded that materials such as tannin-fluoride preparation, polycarboxylate cement and calcium hydroxide were effective in promoting remineralisation and reducing microorganisms in remaining carious tissue. The stepwise excavation was effective in preserving the pulp of asymptomatic teeth presenting with deep carious lesions (Hayashi *et al.* 2011).

Although this two-part clinical technique aims to avoid exposure of the pulp, it still poses a degree of pulp exposure risk in the second excavation in comparison to partial caries removal with no re-entry. The ratio was 56% reduction in risk of pulp exposure with stepwise caries removal in comparison to complete caries removal (Ricketts *et al.* 2013). Severe damage can occur to exposed pulp during the restorative procedure (Murray *et al.* 2002b), thereby affecting defence mechanisms. Exposed cavities were associated with more severe forms of inflammation, and an increased frequency of bacterial microleakage in comparison with non-exposed cavities (Murray *et al.* 2002b). Also, the prognosis of direct pulp-capping of carious exposure is not predictable clinically; moreover, lower risk of pulp exposure is expected in deep carious lesions after 6 months re-entry (Ricketts 2001).

In conclusion, partial caries removal has an advantage in comparison to complete caries removal in reducing pulp exposure by 98% in teeth with deep carious lesions, no evidence of additional pulpal and restorative adverse complications are associated with partial caries removal if well sealed restoration acquired (Ricketts *et al.* 2006, Van Thompson *et al.* 2008). Also, it is apparent that incomplete caries removal can change the ecology and behaviour of carious lesion dramatically without the need for a second excavation if tight coronal seal is acquired in the first appointment. It is essential that future studies focus on the ways that carious dentine is removed during caries excavation procedures, as this may play an important role in decreasing pulp exposure and maintaining pulp vitality.

2.9 Minimal invasive caries removal and caries removal techniques

The main goal of conservative dentistry is to preserve function, aesthetic and health of dentition in an optimum status. Recently attention has been drawn to minimally invasive approaches of conservative dentistry by utilising adhesive technology alongside advances in diagnostic and caries removal techniques (White J.M. and W.S. 2000). Traditionally, dental caries is treated with the idea of an extension for prevention due to the inherent shortcomings of restorative materials used for tooth restoration. Minimally invasive caries removal techniques are among the most significant applications of the minimal intervention dentistry concepts (Murdoch-Kinch and McLean 2003).

Methods used for caries removal include traditional mechanical rotary dental burs utilised with high and low-speed handpieces, chemo-mechanical caries removal, laser, air-abrasion and sono-abrasion, Excavation Aided by Laser-induced Fluorescence and Fluorescence-aided Caries Excavation "FACE" (de Almeida Neves *et al.* 2011).

2.9.1 Mechanical rotary dental burs

Carious / non-carious dentine removal is usually achieved by conventional mechanical rotary burs. Firstly in 1871, James Morrison introduced metal hand drills, later in 1947 low-speed rotary instruments were introduced with a rotation speed of 10,000 rpm, followed by high-speed instruments in 1957 with a rotation speed up to 250,000 rpm (Roberson *et al.* 2006). Although the subsequent development of high-speed rotary instruments resulted in efficient caries removal process, it resulted in the collateral destruction of healthy tooth structure (Yip and Samaranayake 1998). There are many disadvantages associated with the usage of rotary instruments, such as heat generation (thermal stimulation), pressure and vibration (mechanical stimulation), non-selective removal of carious dentine and excessive removal of healthy tooth structure and need of local anaesthesia in addition to noise (Banerjee *et al.* 2000b).

Heat generation during cavity preparation could cause permanent damage to the pulp. It has been found that 40% of subjects suffered from irreversible inflammation in the pulp when there was 5.5°C increase in the pulpal temperature and there was pulp necrosis when there was 11°C increase in pulpal temperature above normal temperature (Zach and Cohen 1965). During the high-speed procedure, air-water spray cooling is vital regardless of the pressure applied or type of the bur used (Lauer *et al.* 1990). To prevent excessive desiccation adequate cooling is essential, also to facilitate drilling efficiency with diamond points, or steel or carbide burs (Taira *et al.* 1990). Another study found that a combination of high speed, controlled temperature and a light load is favourable to minimise pathological alterations in the pulp (Stanley and Swerdlow 1959). Cutting of carious dentine by rotary burs will lead to the opening of healthy dentinal tubules along with water stimulation of the nerve fibres attached to the odontoblastic processes, which will cause pain associated with cavity preparation (Banerjee *et al.* 2000c). The pain associated with rotary burs drilling requires local anaesthesia, which can be considered the main discomfort of the patient in the dental chair along with noise and vibration associated with rotary burs cavity preparation. Also, over-excavation is usually associated with the use of rotary dental burs in comparison to other excavation methods, due to lack of tactile control, which results in the removal of healthy tooth structure (Banerjee *et al.* 2000b, Celiberti *et al.* 2006).

2.9.2 Chemomechanical caries removal

Chemo-mechanical caries removal methods were introduced as an alternative to conventional rotary mechanical methods for caries removal, to prevent the unnecessary removal of viable, healthy tooth structure. The removal agents are either sodium hypochlorite- (NaOCl-) or enzyme-based; the NaOCl-based agents include GK-101, GK-101E (Caridex) and Carisolv, and the enzyme-based agents include Papacarie and Biosolv (Hamama *et al.* 2014a).

2.9.2.1 NaOCl-based agents

Many products have been introduced since 1975 when Habeb *et al.* reported an alternative to rotary carious tissue excavation; chemical carious tissue removal using 5% sodium hypochlorite (Habib *et al.* 1974). However, this concentration was toxic; therefore, there have been attempts to modify the formula by adding sodium hydroxide, sodium chloride and glycine to the 5% sodium hypochlorite. This modified formula was known as GK-101 and it was composed of N-monochloroglycine (Goldman and Kronman 1976). Caridex™ was later developed from a formula made of N-monochloroglycine and amino butyric acid (Schutzbank *et al.* 1978). Caridex is associated with a high treatment cost, requires preheating, is time-consuming, requires special delivery apparatus and has a short shelf life (Barwart *et al.* 1991). Therefore, Carisolv™ gel (MediTeam Dental; Sävedalem, Sweden), was introduced in 1998 in an attempt to solve some of these problems. A summary of chemo-mechanical caries removal products is provided in Table 2-2 (Hamama *et al.* 2014a).

Table 2-2: Summary of chemical formula and mode of action of chemo-mechanical caries removal systems (Hamama *et al.* 2014a).

CMCR agent	Chemical formula	Mode of action	Year of production	Manufacturer
GK-101	Solution A: 0.05% N-monochloroglycine. Solution B: 4-6% NaOCl.	Chlorination of the denatured collagen by conversion of hydroxyproline (essential factor of the stability of collagen) to pyrrole-2-carboxyglycine.	1976	National Patent Dental Products, Inc., New Brunswick, NJ, USA.
GK-101E (Caridex)	Solution A: N-monochloro-DL-2 amino butyrate (NMAB) Solution B: 4-6% NaOCl	Same as GK-101 (chlorination mechanism) + cleavage of the denatured collagen fibrils as a result of the oxidation of glycine residues	1984	National Patent Dental Products, Inc., New Brunswick, NJ, USA
Carisolv™	Original gel (before 2004): Syringe A: carboxymethylcellulose-Based gels, colouring agent and amino acids (glutamic, leucine and lysine) in one Syringe B: 0.25% NaOCl in the other. Modified gel (after 2004) Multimix syringe: the red colouring agent was removed, the amino acid concentration was reduced by half and the NaOCl concentration was increased to 0.475%.	Similar to Caridex, except the replacement of monoaminobutyric acid by three different charged amino acids. These acids were shown to react with different moieties of a carious lesion.	1998, then modified in 2004, and in 2013 the New Carisolv System© was introduced	Medi Team Dentalutveckling AB, now Rubicon Life Science AB, Göteborg, Sweden
Papacarie	Papain enzyme, chloramine, toluidine blue, salts, preservatives, a thickener, stabilisers and deionised water.	The action of Papacarie was the result of degradation of proteoglycans of Dentinal matrix.	2003	Formula & Acao, Brazil
Biosolv (SFC-V and SFC-VIII)	Pepsin enzyme in a phosphoric acid/sodium biophosphate buffer.	Phosphoric acid dissolves the inorganic components of caries-infected dentine; while permitting the pepsin enzyme to selectively disrupt the denatured collagen fibres.	2006	3M-ESPE AG, Seefeld, Germany

Carisolv™ originally utilised a mixture of sodium hypochlorite in one syringe and three amino acids (lysine, leucine and glutamic acid) in a carboxymethylcellulose gel preparation in another syringe, with erythrocin dye to make it readily visible (Sakoolnamarka *et al.* 2005, de Almeida Neves *et al.* 2011). Later the gel was reformulated, a higher concentration of NaOCl was used and the colouring agent has been removed to make the gel colourless (Nr Clementino-Luedemann *et al.* 2006).

Figure 2-4: Carisolv™ gel (twin syringe system)

The mixture has a pH of 11, its mechanism of action depends on the chlorination of amino acids by sodium hypochlorite, chloramines assumed to disrupt hydrogen bonds in cross-links of collagen of carious dentine matrix resulting in easy removal with hand instruments (Banerjee *et al.* 2000c, Venkataraghavan *et al.* 2013, Beeley *et al.* 2001). Even though sodium hypochlorite is a non-specific deproteinising agent that can attack collagen matrix of sound dentine, the mixture of amino acids intended to reduce the aggressiveness of sodium hypochlorite by a buffering effect to limit the disruption effect of collagen bonds only to the deteriorated collagen of carious dentine and selectively affect the infected carious dentine only (Tonami *et al.* 2003).

Mechanism of action of Carisolv^{TM} gel

Dentine consists of 70%, 10% and 20% of minerals, water and organic matrix respectively. The organic matrix contains 90% collagen and 10% non-collagenous components (Hall and Embery 1997). Collagen contains large amounts of proline and glycine, and it is structured as fibrils formed of tropocollagen units which are triple helixes formed from coiled polypeptide chains. Stability of collagen fibrils come from the cross-link of covalent bonds between the polypeptide chains and the covalent bonds between the tropocollagen units (Figure 2-3) (van der Rest and Bruckner 1993). When caries reaches dentine, complete/partial demineralisation and degradation of organic matrix occur because of bacterial proteases and other hydrolyses (Thylstrup and Fejerskov 1994). Carisolv^{TM} reagent cleaves the cross-linkage in polypeptide chains in the triple helix and causes additional degradation of partially degenerated collagen as explained in Figure 2-4.

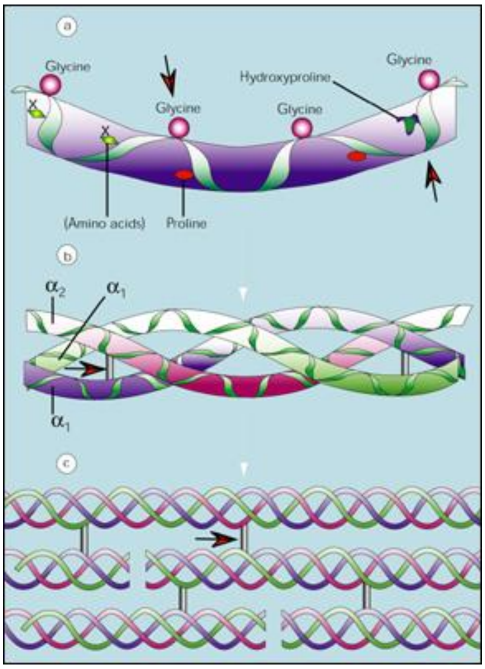


Figure 2-5: Diagram of collagen cross linkage a, Polypeptide chain. Possible sites of cleavage by Carisolv^{TM} reagents by degradation of glycine or hydroxyproline are indicated by red arrows. b, triple helix. Sites of cleavage by degradation of intra-molecular cross links are shown by red arrows. c, Tropocollagen units assembled to form a collagen fibril. Sites of cleavage by degradation of intermolecular cross links are indicated by red arrows. Source Beeley *et al.* (2001).

Hand instruments of Carisolv^{TM} system:

Special hand instruments as shown in Figure 2-6 designed to have access to the different lesions in order to remove the softened carious tissue gradually. These instruments are characterised by an effective cutting ability to a controlled depth, because they have blunt cutting angles and sharp edges causing an area of support to the underlying surface. In contrast other sharp cutting instruments such as drills and excavators cutting characterised by less area of support and aggressive cutting angles as represented in Figure 2-6.

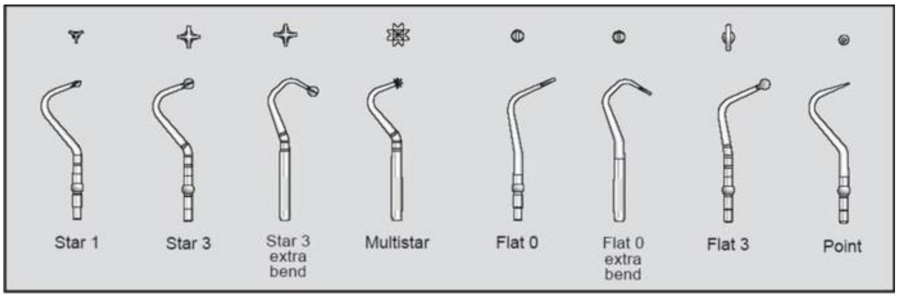


Figure 2-6: Carisolv^{TM} instruments with different tips to access different types of lesions.Adaptedfromhttp://archieve.ijds.in/functions.php?fuse=23&SrNo=23&CurrentIssue=No&IssueVol=Vol.%205&IssueNumber=Issue%205&ArticleID=583

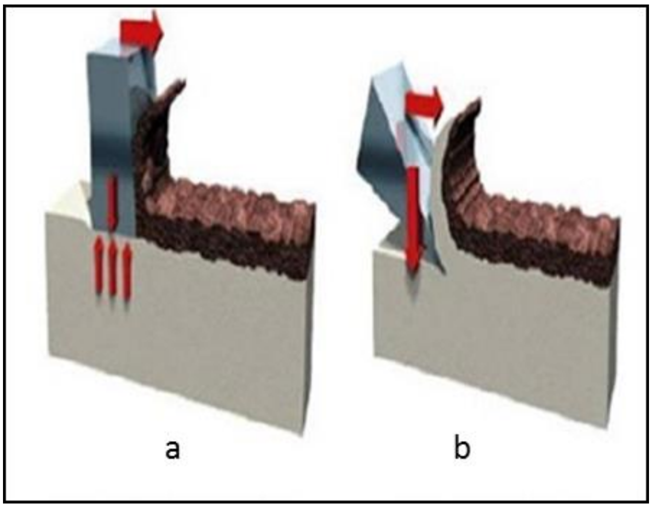


Figure 2-7: Representative diagram of the proposed mode of action of Carisolv^{TM} instruments versus burs and hand excavators in caries removal. a) Carisolv^{TM} instruments tips have sharp edges but a blunt angle. They provide excellent depth control when scraping away the softened carious dentine by the gel. b) Rotary burs and traditional hand excavators with sharper cutting angles are designed to work themselves down into dental tissue and make it difficult to control the depth of excavation.

Comparison between Carisolv™ and mechanical rotary burs method

Compared to conventional mechanical rotary burs removal methods, chemomechanical removal is more time consuming; however, it appears to preserve healthy tooth structure and reduce the need for local anaesthesia administration (Banerjee *et al.* 2000b, Munshi *et al.* 2002, Kakaboura *et al.* 2003, Kirzioglu *et al.* 2007, Venkataraghavan *et al.* 2013). Caries removal with the chemomechanical method was used to standardise the extent of caries removal in clinical studies that compare the effect of different materials on dentine-pulp complex in teeth with deep caries, due to its ability to selectively remove caries-infected dentin only and maintain the caries-affected dentine layer (Hashem *et al.* 2015).

Many studies have been conducted to assess the effectiveness (extent of caries-affected dentine preservation) and efficiency (time taken to prepare cavity) of chemo-mechanical caries removal in comparison to conventional rotary burs; these concluded that chemo-mechanical caries removal is more effective and less efficient compared to rotary burs (Sakoolnamarka *et al.* 2005, Banerjee *et al.* 2000b, Flückiger *et al.* 2005). Carisolv™ needs more time to accomplish the procedure which includes gel application into the cavity, scraping off the carious lesion, rinsing and repeat the procedure if necessary, however, it is better accepted by the patients and might not need local anaesthesia (Fure *et al.* 2000, Kakaboura *et al.* 2003, de Almeida Neves *et al.* 2011).

In term of the bond strength of resin bonding agents to the dentine substrate left after excavation either with Carisolv™ or conventional bur excavation, there was no difference in mean shear bond strength between two techniques, no matter what type of the bonding agent to be either 2 step etch and rinse type or self-etch type (Burrow *et al.* 2003, Sattabanasuk *et al.* 2006, Silva *et al.* 2006, Tachibana *et al.* 2008). However, Carisolv™ treatment before acid etching in primary teeth resulted in lower bond strength in comparison to that of permanent teeth (Hosoya *et al.* 2001). In the same context micro-leakage decreased in teeth filled with resin composite after treatment with Carisolv™ due to smear layer free irregular surface that can enhance adhesion to adhesive restorative materials as confirmed by SEM. However, in teeth with deep caries that have been treated with Carisolv™ , it was suggested to use calcium hydroxide as an anti-bacterial and reparative dentine inducer as there will be a remaining dentinal caries which should be preserved to maintain health of the pulp in these cases (Yamada *et al.* 2006). Also, it has been found that the use of Carisolv™ in combination with DIAGNOdent assessment and caries detector solution may lead to complete removal of caries in comparison to traditional visual and tactile criteria, which is contra-indicated in teeth with deep caries (Yamada *et al.* 2006). Similarly, other study found that Carisolv™ did not increase nano-leakage after bonding to caries-affected dentine with different bonding adhesives and procedures (Kubo *et al.* 2002).

The chemical and mechanical characteristics of dentine after excavation with Carisolv™ did not differ significantly from that of sound dentine, where Ca:P ratio and micro-hardness were comparable to that of sound dentine (Arvidsson *et al.* 2002, Hossain *et al.* 2003, Sakoolnamarka *et al.* 2005, Hamama *et al.* 2013). There were minimal chemically-induced changes of dentine components after carious dentine removal with Carisolv™. However Carisolv™ resulted in lower dentine hardness in comparison to that found after bur excavation (Hamama *et al.* 2013, Hossain *et al.* 2003, Banerjee *et al.* 2010), this confirms the conservative therapeutic approach of Carisolv™ in preserving caries-affected dentine during cavity preparation.

In term of bacterial analysis after Carisolv™ excavation, although there was abundance of bacterial loads left in dentine (36% of teeth were free of bacteria) in comparison to rotary bur excavation (90% of teeth were free of bacteria) (Yazici *et al.* 2003), in this *in-vitro* study the end point of cavity excavation was hard unstained dentine assessed by tactile and visual criteria, however from a clinical point of view it is not unacceptable to leave bacteria in dentine after excavation, especially in the scope of preserving tooth structure (A Banerjee *et al.* 1999, Kidd *et al.* 1993). The reason for higher bacterial load in Carisolv™ treated dentine was explained by the fact that the bacteria pushed into dentinal tubules due to scraping and burnishing of carious dentine by hand instruments used in conjugation with Carisolv™ gel (Yazici *et al.* 2003), enhanced by the absence of smear layer after excavation with Carisolv™ (Banerjee *et al.* 2000a, Hamama *et al.* 2013). In contrast, other studies found that Carisolv™ leaves bacterial amount less or similar to that after bur excavation (A Banerjee *et al.* 1999, Splieth *et al.* 2001, Lager *et al.* 2003, Azrak *et al.* 2004). The Carisolv™ treated cavities displayed a higher reduction in colony forming units after excavation compared to the drill treated cavities. This might be attributed to the antibacterial properties of Carisolv™, which contains chloramines that have an inherent antibacterial effect.

Regarding the biological effect of Carisolv™ on dental pulp, there was a similar pulpal response to both Carisolv™ and sterile saline solution (as a control) after one week of exposure to each of the tested materials, however Carisolv™ gel shows haemostatic effect on the pulp after exposure in comparison to control group and it has no effect on sensory nerves in dental pulp (Young and Bongenhielm 2001, Bulut *et al.* 2004).

2.9.2.2 Enzyme-based agents

New products for chemo-mechanical caries removal were introduced in 2003 in Brazil and are based on papain (Papacarie®) shown in Figure 2-8. This product is composed of papain, chloramines, salt and gel, and has bacteriostatic, bacteriocidal and anti-inflammatory features (Bussadori *et al.* 2006). Mechanism of action of Papacarie® on caries is comparable to the mechanism of Carisolv™ by eradication of fibrin layer of carious tissue which is associated with bubbles because of oxygen release (Bussadori *et al.* 2006).

Similar to Cariosolv™, Papacarie® was effective in reducing patient discomfort, pain and microbial loads in comparison to rotary burs especially in children, particularly those who present with early childhood caries or management problems (Kotb *et al.* 2009, Singh *et al.* 2011, Motta *et al.* 2014, Kochhar *et al.* 2011). Long-term clinical and radiographic follow-up for 24 months of patients showed a success rate of more than 90% (Bussadori *et al.* 2011). Although Gupta et al found high microbial loads after complete caries removal with Papacarie®, which justifies the use of anti-microbial restorative materials to tackle this problem both in permanent and deciduous teeth, however, Papacarie® was able to differentiate between caries-infected and caries-affected dentine clinically using visual and tactile criteria (Gupta *et al.* 2013).

In-vitro resin tag length in teeth treated with Papacarie® were longer and significantly superior to those in teeth treated with rotary burs (Arora *et al.* 2012). However, Papacarie® resulted in lower micro-tensile bond strength in teeth restored either with GIC or resin composite in comparison to that found in teeth excavated with rotary burs (Wahby *et al.* 2014). Experimentally Carisolv™ needs more time than Papacarie® in removing caries; however, teeth excavated with Carisolv™ show better marginal seal and shear bond strength to resin composite in comparison to teeth excavated with Papacarie® (PM Viral *et al.* 2013). Furthermore, both Papacarie® and Carisolv™ did not adversely affect bonding of the self-etch adhesive to caries-affected dentine (Hamama *et al.* 2014b).

Teeth excavated with Papacarie® showed a significantly higher reduction in the number of bacteria in comparison to those excavated with Carisolv™ and rotary burs (El-Tekeya *et al.* 2012), but other studies found a similar amount of reduction in the number of bacteria using Papacarie® and Carisolv™ (Kush *et al.* 2015). Moreover, both products showed *in vivo* and *in vitro* adequate biocompatibility (Martins *et al.* 2009).

Figure 2-8: Papacarie® gel (Formula & Acao, Brazil).

In 2006 3M-ESPE AG introduced Biosolv (SFC-V and SFC-VIII) which is a pepsin enzyme based chemo-mechanical caries removal system. It is composed of Pepsin enzyme in a phosphoric acid and sodium biphosphate buffer prepared in a gelling agent, which is used with a plastic hand instrument (STAB VI.3), which could not remove dentine by itself (Nr Clementino-Luedemann *et al.* 2006). Its mechanism of action depends on pepsin's ability to dissolve denaturated but not sound collagen in dentine. Collagen-polymer in the superficial non-helical collagen is split by the Pepsin into a monomer (Tonami and Ericson 2005, Nr Clementino-Luedemann *et al.* 2006).

Micro-computed topographical analysis showed that both Carisolv™ and SFC-V were able to remove the superficial most demineralised layer of carious dentine comparably even without stainless steel mechanical intervention, however there was a significant difference in morphological texture of the resulting dentine surface after each chemomechanical excavation material. Carisolv™ resulted in rough, non-organised layer of dentine, while SFC-V resulted in an organised layer with open dentinal tubules, which may be attributed to the effect of acid in the enzyme preparation, which dissolves the remnants of hydroxyapatite during treatment leding to no smear layer formation (Nr Clementino-Luedemann *et al.* 2006).

The use of stainless steel made instrument with Carisolv™ and SFC-V lead to a comparable amount of carious dentine removal, which was detected by novel confocal fiber-optic microendoscopy to detect auto-fluorescence signals from the original and residual caries. Carisolv™ excavation resulted in lower auto-fluorescence signal from the remaining dentine in compare to SFC-V which suggest under –preparation with SFC-V excavation (Banerjee *et al.* 2010).

2.10 Materials for pulp capping

Pulp protection from additional bacterial, chemical and thermal insults represent a prerequisite for restorative treatment. The most suitable barrier is natural dentine either the primary existing dentine or the reactionary/reparative dentine that formed by the pulp odontoblasts due to the function of therapeutic pulp capping liners (Hilton 2009)

A range of materials with bacteriostatic, bactericidal, or remineralizing properties on the defected dentine are applied before the restoration.

2.10.1 Calcium Hydroxide

Calcium hydroxide was first introduced in dentistry in 1920 by Herman (Hermann 1920), it's bacteriostatic, biocompatible and its stimulation of dentine bridge formation drive its use as direct and indirect pulp capping agent also it is used in apexogenesis, apexification, treatment of root resorption, and inter-appointment intra-canal dressing and it was considered the gold standard material for direct and indirect pulp capping (Baume and Holz 1981, Weiner 2002, Farhad and Mohammadi 2005).

The mechanism of calcium hydroxide in promoting pulp healing and reactionary/reparative dentine formation in direct/indirect pulp capping is not well known but it has been suggested to be associated with its high alkalinity achieved via the release of hydroxyl and calcium ions from calcium hydroxide (Estrela 1994). Ions released from calcium hydroxide are responsible for its high alkaline pH, however, high pH of calcium hydroxide causes coagulation necrosis when it comes in contact with exposed pulpal tissue (Holland 1971). Studies have shown that this superficial pulp necrosis forms calcium carbonate globules which act as dystrophic calcification nucleus within dense reticular fiber deposition layer (Holland 1971), followed by odontoblast-like cells layer that differentiates to produce dentine, therefore superficial pulpal necrosis is essential for healing of pulpal tissue promoted by calcium hydroxide (Pereira *et al.* 1980). Although hydroxyl ions are responsible for high alkalinity which is bactericidal, however, it is not the sole initiator of the healing process in the pulp (Farhad and Mohammadi 2005). Recently it has been found that the release of growth factors and other bioactive molecules from the dentine by calcium hydroxide will mediate the cellular activities involved in dentine bridge formation (Graham *et al.* 2006).

Calcium hydroxide is an active inducer of dentine formation when it comes in direct/indirect contact with pulpal tissue in comparison to other pulp protection materials such as varnishes, adhesive resins, resin composites and glass ionomer cement (Modena *et al.* 2009). Calcium hydroxide ability to induce reactionary/reparative dentine formation with its bacteriostatic activity in direct/indirect pulp capping has been demonstrated by many studies (Pereira *et al.* 1980, Holland 1971, Siqueira and Lopes

1999, Zehnder *et al.* 2006). Clinically calcium hydroxide shows high success rate in direct/indirect pulp capping over an observation period ranging from 2-10 years (Hørsted *et al.* 1985, Matsuo *et al.* 1996, Robertson *et al.* 2000, Accorinte *et al.* 2006). Also in inflamed pulps calcium hydroxide demonstrated the ability to promote healing of these pulps by reducing inflammation and formation of hard tissue at the exposed site (Stanley and Pameijer 1997).

However, calcium hydroxide's long-term solubility is a reason for concern. These materials are soluble in water and acids and dissolve clinically after 1-2 years leading to microleakage (Schuurs *et al.* 2000). Calcium hydroxide drawbacks are degradation by acids during etching in adhesive bonding and lack of mechanical/chemical adhesion to dentine (Cox *et al.* 1999). In addition, it has a very low compressive strength which can minimise its use as a base material in direct/indirect pulp capping, however recently urethane dimethacrylate with initiators and accelerators has been incorporated with calcium hydroxide to increase its binding and mechanical properties (Schuurs *et al.* 2000). Moreover, dentine bridging after direct pulp capping with calcium hydroxide is characterised by multiple defect tunnels, which permit oral contaminants, bacteria and their toxins to reach the pulpal tissue, thus they fail in providing a long-term biological barrier against bacterial infections (Stanley and Pameijer 1997). A study on monkeys' teeth capped with calcium hydroxide found a significant number of formed dentine bridges contain tunnel defects associated with pulpal inflammation or necrosis with inflammatory and bacterial cells infiltration (Cox *et al.* 1996).

2.10.2 Mineral trioxide aggregate

Mineral trioxide aggregate (MTA) was developed in 1993 (Torabinejad *et al.* 1993), it is a tri-calcium silicate based cement and recommended initially as a root-end filling material and later has been used for pulp capping, pulpotomy, apical barrier formation in teeth with open apexes, repair of root perforations, and as a component of root canal filling materials (Primus 2014). MTA has been recognised as a bioactive (apatite-forming) material when it is exposed to physiologic saline (Enkel *et al.* 2008, Gandolfi *et al.* 2014), also it is tissue conductive, and biocompatible (Moretton *et al.* 2000, Ribeiro *et al.* 2006). Moreover, it has low solubility (Fridland and Rosado 2005) and possesses some antibacterial activity (Zhang *et al.* 2009).

MTA powder contains fine hydrophilic particles composed of dicalcium silicate and tricalcium silicate that set in the presence of moisture. MTA is produced in two types grey and white, with reduced amounts of iron, aluminium, and magnesium present in white MTA than in the grey (Song *et al.* 2006, Parirokh and Torabinejad 2010). Both white and grey MTA are constituted mainly of dicalcium silicate, tricalcium silicate, tricalcium aluminate and tetra calcium alumina-ferrite (Islam *et al.* 2006a). However, the particle size is smaller in white MTA in comparison to grey MTA (Duarte *et al.* 2003). The first formula of MTA was composed of 4:1 Portland cement: bismuth oxide mixture commercialised as ProRoot MTA (Dentsply, Tulsa, OK, USA) (Torabinejad and White 1995). Although it may be concluded that Portland cement can substitute the use of MTA, they are non-identical materials (Roberts *et al.* 2008). In comparison to Portland cement, MTA products have longer working time, contain less toxic heavy metals and have a smaller particle size as they go through a further purification process (Islam *et al.* 2006b).

Hydration of MTA powder results in a colloidal gel that solidifies into a hard structure, The precipitated calcium produces calcium hydroxide which is the cause of MTA’s high alkalinity after hydration (Camilleri 2008). Hydration reaction of tri-calcium silicate and di-calcium silicate is illustrated in equations 2.1 and 2.2, the reaction of calcium hydroxide with phosphate ions body containing fluid will result in precipitation of hydroxyapatite crystals which are the key in bioactivity of MTA (equation 2.3) (Primus 2014).

Tri-Calcium Silicate: $2Ca_3SiO_5+7H_2O=3CaO_2.SiO_2.4H_2O+3Ca(OH)_2$ ……………..(2.1)

Di-Calcium Silicate: $2Ca_3SiO_5+5H_2O=3CaO.2SiO_2.4H_2O+ Ca(OH)_2$……………….(2.2)

Hydroxyapatite precipitation: $7Ca(OH)_2+3Ca(H2PO4)2 \longrightarrow$

$Ca_{10}(PO_4)6(OH)_2+12H_2O$......(2.3)

Both white and grey MTA possess antibacterial and antifungal properties due to its high alkalinity (Modena *et al.* 2009). Calcium ions released from MTA also contribute to the precipitation of hydroxyapatite at the MTA-dentine interface by filling the microscopic space in the interface. This mechanical seal followed by diffusion controlled reaction between the apatite layer and dentine leads to a chemical bonding (Sarkar *et al.* 2005). Another study found the formation of tag like structures extending from the formed apatite layer to the dentine tubules indicating that constant formation of the precipitate contributes not only to the formation of the interfacial layer but also to the promotion of an intra-tubular mineralisation process (Reyes-Carmona *et al.* 2009).

At the cellular level, MTA was more able than calcium hydroxide in initiating hard tissue formation and reducing inflammation by inducing recruitment and differentiation of pulpal stem cells (Holland *et al.* 2001, Aeinehchi *et al.* 2003, Farsi *et al.* 2007, Nair *et al.* 2008). Histological examination of teeth with irreversible pulpitis treated with MTA revealed vital and inflammation free teeth with complete dentine bridge formation (Eghbal *et al.* 2009).

MTA induces the differentiation of odontoblast-like cells from dental pulp cells and enhances tissue healing by promoting the secretion of vascular endothelial growth factor (Paranjpe *et al.* 2010). Interestingly studies suggest that MTA has a favourable biological performance by initiating regenerative reaction of dental pulp through mechanisms like inflammation, repair and hydroxyapatite formation (Modena *et al.* 2009). Recently a new mechanism proposed for the action of MTA in initiating regenerative pulp response by initiation of secretion of dentine extracellular bioactive molecules or components (i.e. growth factors), which are incorporated within extracellular matrix of dentine during primary dentinogenesis, that can drive the regenerative process in the dental pulp (Tomson *et al.* 2007). Therefore MTA placement on dentine remaining layer rather than direct placement on dental pulp tissue may enhance MTA's role in pulp regeneration. In this context, indirect pulp capping can be expected to initiate a better pulpal response in comparison to direct pulp capping, which might result in direct exposure of pulp tissue to the cytotoxic effect of the capping materials. Clinically, the presence of hard tissue barrier can be considered as an advantage to prevent bacterial and chemical products infiltration to the pulpal tissue (Holland *et al.* 1979).

Advantages of MTA over calcium hydroxide products include its very low solubility and its lower cytotoxicity (Poggio *et al.* 2014). In animals, MTA resulted in remarkable success compared to calcium hydroxide (Ford *et al.* 1996, Holland *et al.* 2001). Mouse MDPC-23 odontoblast-like cells and OD-21 undifferentiated pulp cells did not experience apoptosis in any of the cell lines. WMTA was reported to cause a profound effect on cells proliferation by increasing DNA synthesis (Moghaddame-Jafari *et al.* 2005). It has been found that calcium release from MTA has a significant effect on the proliferation of human dental pulp cells, MTA stimulates cell proliferation significantly after 12 days compared to calcium hydroxide which has no such effect (Takita *et al.* 2006).

Histological studies showed that teeth pulps capped with MTA have fewer signs of inflammation and more homogenous, thicker and faster dentine bridge formation after 4, 8 weeks and 6 months in comparison to that capped with calcium hydroxide (Aeinehchi *et al.* 2003, Chacko and Kurikose 2006, Accorinte *et al.* 2008), however, another study found minimal clinical and histological differences between both materials after 6 months examination (Iwamoto *et al.* 2006).

Clinically MTA shows higher long-term success rate in comparison to calcium hydroxide in direct pulp capping, the success rates were 78% and 60% respectively after 2 years period (Mente *et al.* 2010). Likewise in a practice-based research network, 2yr randomised clinical trial on direct pulp capping, MTA shows a lower probability of failure compared to calcium hydroxide (19.7% and 31.5% respectively) (Hilton *et al.* 2013). However, the probability of failure increase significantly in teeth capped with MTA by undergraduate students after 2 years recall, certain pre-operative variables such as patient age, exposure size and bleeding time were found to be not predictive of clinical outcome in these cases (Miles *et al.* 2010). In contrast, a nine years observational study on permanent teeth capped directly with MTA by experienced clinician shows very high clinical success rate (97.96%) in teeth with reversible pulpitis (Bogen *et al.* 2008). Similarly, indirect pulp capping with MTA resulted in 89.9% clinical success compared with 73% with calcium hydroxide after 6 months observational period (Leye Benoist *et al.* 2012). Obviously, pulp capping with MTA may be considered a treatment option with a predictable prognosis in teeth with signs and symptoms of reversible pulpitis with high clinical success rate. However, there are concerns regarding the discoloration after MTA placement especially in anterior teeth for aesthetic reasons. It has been found that there was significant discoloration in extracted premolars measure by spectrophotometer after 12 weeks of MTA placement compared with Endocem (new Pozzolan cement) *in-vitro* (Jang *et al.* 2013). Therefore, vital pulp therapies in anterior teeth using MTA may need to be reconsider unless an effective method is available for reverse this discoloration.

The average setting time of white MTA is 2 hours 45 minutes, which appears to be the material's primary disadvantage for clinical use (Neelakantan *et al.* 2012). Researchers aim to reduce the setting time of MTA by adding calcium chloride as an accelerator or by the elimination of calcium sulphate as a retarder of setting reaction (Primus 2014, Bortoluzzi *et al.* 2009). Also, it has poor mechanical and handling properties that reduce the chance to restore the pulp capped tooth definitively in one appointment in order to provide a good seal as it will lose its consistency in presence of excess liquid (Islam *et al.* 2006b). MTA has a reported delayed setting time from 75 minutes up to 73 hours (Chng *et al.* 2005) Therefore it has been used in conjugation with materials including glass ionomer cement (GIC) or resin-modified glass ionomer cement (RMGIC) to provide a mechanical barrier until maturation, thus requiring a two-step clinical procedure (Rada 2013). Light cured MTA has a reduced setting time (20 seconds of light curing, for a 1.7mm thickness of the material) which may avoid the risk of untimely dissolution but it is not as osteoconductive as conventional MTA (Gomes-Filho *et al.* 2008, Gandolfi *et al.* 2012). One of the new commercially available MTA products is MTA®caps (Acteon, Pierre Rolland, Merignac France) which is injectable and well suited for pulp capping procedures due to its fast clinical setting time in 10 minutes according to manufacturer information. A study reported the chemical compositions and setting kinetics of different MTA products. MTA®caps was found to have the lowest shear modulus G' (elastic) among other products of MTA with 0.7 Gpa shear modulus G' after 150 min. Chemical analysis with Inductively Coupled Plasma-Atomic Emission Spectroscopy and X-ray Energy Dispersive analysis revealed calcium tungstate as a radio-opacifier in this product with traces of chlorine (Setbon *et al.* 2014).

Pulp capped teeth must be sealed using restorative materials such as glass ionomer cement (GIC) or resin composite. Therefore the bond and seal between restorative materials and pulp capping agents are important and if there is a lack of adequate seal, the intrusion of bacteria into pulp and failure of the pulp capping procedure will occur (Paterson 1976, Bergenholtz 2000, Wells *et al.* 2002, Tselnik *et al.* 2004). Many clinicians believe that resins should not be placed directly on top of freshly mixed MTA, this is because the etching and bonding protocols may affect the setting reaction of the MTA and thereby cause it to disintegrate (Nandini *et al.* 2007, Yesilyurt *et al.* 2009). Appreciating the effect of bonding of resin-based composite or GIC to MTA is clinically relevant. Determining the bond strength between the two materials gives the clinician a fair idea about the quality of restorations. (Neelakantan *et al.* 2012).

2.11 Bacterial spectrum of dental caries

Dental caries is the most common bacterial-related disease in the world, in fact, 36% of world's population have dental caries lesions in their permanent teeth (Anusavice 2002, Vos *et al.* 2012). The main three hypothesis of the development of dental caries from a bacteriological point of view includes a specific plaque hypothesis (Loesche 1992), a non-specific plaque hypothesis (Theilade 1986) and ecological plaque hypothesis (Marsh 1994).

The specific plaque hypothesis proposes that selected species of bacteria are involved in the initiation and progression of a disease such as *Streptococcus mutans* and

Streptococcus sobrinus similar to most infectious diseases. On the other hand, the non-specific hypothesis suggests that dental caries is the result of the total activity of total plaque microflora, the accumulation and proliferation of plaque above the threshold of host resistance will initiate the disease status regardless of the type of bacterial species in the dental plaque.

The ecological plaque hypothesis suggests that there is a dynamic relationship between dental plaque microbiota, saliva and diet in the pathobiology of dental caries. It suggests that caries is a dietary carbohydrate-modified bacterial infectious disease caused by a dietary carbohydrate-induced enrichment of *Streptococci and Lactobacilli* or other bacteria in the dental plaque which will increase the cariogenic potential and lower pH of the dental plaque significantly (Van Houte 1994). Other species of bacteria have been suggested to be a secondary pathogen in dental caries such as *streptococcus sorbinus*, both species can produce lactic acid which causes dental caries. In addition to many *Lactobacilli* species and *Actinomyces* species also suggested playing a part in dental caries especially root surface caries (Van Houte 1994). In normal conditions, the balance between de and remineralisation processes in supragingival biofilm do not cause any harmful effect on teeth, however homeostasis in the biofilm may break down in case of excessive carbohydrate enrichment diet or low salivary clearance of food leading to change in biofilm microbiota composition and imbalance toward demineralisation and caries (Marsh 1994). Ecological disturbance in the composition of biofilm usually described to be acid induced adaptation and selection processes which lead to domination of aciduric and acidogenic bacteria such as *Streptococci, Lactobacilli, Actinomyces and Bifidobacteria* reflecting the complex nature of aetiology of dental caries (Takahashi and Nyvad 2008, Takahashi and Nyvad 2011).

In the dental plaque biofilm, there are different types of bacterial pathogens, commensals and mutualist, all together they compose the microbiome of the dental biofilm. Investigators have realised that to fully understand human health and disease, there is a need to understand not just a few suspected pathogens, but rather all members of the microbiota (Dewhirst 2016).

However, most investigations of dental caries microbiota have been undertaken using culture methods, which cannot identify many bacteria (Becker *et al.* 2002, Nyvad *et al.* 2013). Molecular methods of bacterial identification such as DNA sequence-based assays can identify bacteria which may not grow on culture media or they are not distinguishable from similar species phenotypically (Hugenholtz and Pace 1996, Pace *et al.* 1986, Becker *et al.* 2002). About two-thirds of oral bacteria have been cultured using traditional methods, some of the uncultured bacteria need particular nutrients whereas others may be suppressed by by-products from other bacteria and /or substances in the culturing media. The interaction between bacterial species is essential for the growth of bacteria in complex biofilm especially in the oral cavity (Wade *et al.* 2016). Around 620 predominant oral bacterial species were identified by molecular techniques 35% of which have not yet been cultured *in vitro* (Dewhirst *et al.* 2010, Dewhirst 2016). However, recent advances in sequencing techniques such as 454 pyrosequencing have discovered greater complexity in the human oral microbiome, where about 10000 phylotypes isolated from pooled dental plaque and saliva samples of 98 and 71 subjects respectively which is higher than previously reported using the traditional sequencing technology (Keijser *et al.* 2008).

16S ribosomal RNA gene (16S rRNA), is a component of the 30S small subunit of prokaryotic ribosomes. This gene can be analysed after DNA extraction from the bacterial samples, it has been used for bacterial phylogeny because of the low chances of mutation of this essential molecule in the bacterial ribosome. This 16S rRNA method allows identifying other uncultivable bacteria in the oral microbiome (Dewhirst 2016).

The enumeration of bacterial loads using qPCR method by TaqMan universal primers quantifies the number of the 16S rRNA genes in the sample rapidly, by detecting a fluorescent signal during each round of amplification allowing enumeration of 16S rRNA genes without the need for post-PCR processing (Heid *et al.* 1996). In addition, a large number of samples can be processed using the inbuilt 96-well format at the same time. However, there is a caveat related to this method, because each bacterium has different numbers of 16S rRNA gene from one species to another (Farrelly *et al.* 1995). Therefore the total number of 16S rRNA genes does not represent the exact number of bacterial cells in the sample. However, in complex bacterial communities in health-related situations, samples may contain impurities and/or matrix substances with multiple species of bacteria, which make other techniques much less sensitive and precise compared to this technique, which provides a fast and reliable method of bacterial enumeration (Nadkarni *et al.* 2002). Studies have used this method to detect bacterial numbers in carious dentine compared to the conventional culture methods and found significantly greater numbers of bacteria yielded from PCR compared to the culture methods, especially in determining anaerobic bacterial counts (Martin *et al.* 2002, Nadkarni *et al.* 2002).

Various molecular studies investigated dentine caries using different molecular techniques found that these lesions were usually dominated by *Lactobacillus* in the advanced front of the lesion (Byun *et al.* 2004, Chhour *et al.* 2005, Munson *et al.* 2004, Obata *et al.* 2014). Other studies reported that phylotypes of the genera *Prevotella, Selenomonas, Dialister, Fusobacterium, Atopobium, Olsenella, Bifidobacterium* and *Pseudoramibacter* have also been commonly identified (Martin *et al.* 2002, Chhour *et* *al.* 2005, Lima *et al.* 2011). Other pyrosequencing studies found that *Cryptobacterium, Lactobacillus, Megasphaera, Olsenella, Scardovia, Shuttleworthia, Cryptobacterium* and *Streptococcus* (Jiang *et al.* 2014), or *Prevotella, Lactobacillus, Selenomonas* and *Streptococcus* were in significantly increased levels in dentine caries samples (Schulze-Schweifing *et al.* 2014). Variation in detected spectrum of the microbiome in dentine caries can be attributed to the various molecular methods used, different sampling procedures, ethnical variations and various histopathological statuses of the inspected lesions.

In a study on children with and without early childhood dental caries (ECC) using 16S ribosomal DNAs were used to identify bacterial species associated with early childhood caries. Plaque samples collected and analysis performed with cloning and sequencing of bacterial 16S ribosomal DNAs. It was found that some species, such as *Streptococcus sanguinis* were associated with health, while others, such as *S. mutans*, other *Streptococcus spp*., *Veillonella spp*., *Actinomyces spp*., *Bifidobacterium spp.*, and *Lactobacillus fermentum* were associated with caries. In addition, ten novel phylotypes were identified. (Becker *et al.* 2002). Similarly, another study on children with/without ECC using 16S RNA gene sequence method and reverse-capture checkerboard hybridization found that most abundant bacterial species associated with the caries-active group were *Actinomyces spp., Streptococcus mutans and Latobacillus spp.*which exhibited inverse relationship to other bacterial beneficial species such as *Streptococcus parasanguinis, Abiotrophia defective, Streptococcus mitis, Streptococcus oralis and Streptococcus sanguinis (Corby et al. 2005).* These results support the fact there are some bacterial species associated with health status compared with other bacterial species associated with disease status in children with/without dental caries.

In adult patients with dental caries, a study conducted to identify bacterial species in the middle part and front of the dentinal carious lesion using 16s rRNA gene sequence analysis and culture methods (aerobic and anaerobic conditions), molecular method detected 31 novel taxa in dental caries. There was no statistically significant difference between middle part and front of the lesion. The predominant taxa via anaerobic cultivation were the novel *Propionibacterium sp.* (18%), *Olsenella profusa* (14%), and *Lactobacillus rhamnosus* (8%). The predominant taxa in the molecular analysis were *Streptococcus mutans* (16%), *Lactobacillus gasseri/johnsonii* (13%), and *Lactobacillus rhamnosus* (8%) (Munson *et al.* 2004). Furthermore, another study was conducted to identify bacterial diversity in coronal dental caries in teeth with chronic pulpitis without carious exposure using anaerobic, microaerophilic conditions and real-time PCR. *Lactobacilli* and *Prevotella spp.* were the most abundant bacteria in the culture method. Real-time polymerase chain reaction (PCR) results revealed greater loads of anaerobes (41 folds) compared to culture colony counting in general. Some species such as *Prevolella* and *Fusobacterium spp.* showed higher loads (82 and 2.4 folds respectively) by real-time PCR compared to colony counting. Also, correlation matrices of real-time PCR data show a positive association between *Micromonas micros* and *Porphyromonas endodontalis* detection and inflammatory degeneration of pulpal tissues (Martin *et al.* 2002).

Furthermore, another study was conducted to identify bacterial species diversity in advanced (deep) caries in adults patients using real-time PCR analysis using universal primers for the 16S rRNA gene, PCR amplicons were cloned and approximately 100 transformants were processed for each lesion. A diverse array of *lactobacilli* was found to comprise 50% of the species, with *Prevotellae* also abundant, comprising 15% of the species. Other taxa present in a number of lesions or occurring with high abundance included *Selenomonas spp., Dialister spp., Fusobacterium nucleatum, Eubacterium spp.*, members of the *Lachnospiraceae* family, *Olsenella spp., Bifidobacterium spp., Propionibacterium sp., and Pseudoramibacter alactolyticus*. They did not find that *Steptococcus mutans* as the most abundant species in contrast to other studies (Chhour *et al.* 2005). Moreover, another study involving primary and permanent teeth in children and young adults was conducted to detect all bacterial species associated with dental caries in primary and permanent teeth at different stages of the disease. Both healthy individuals and those with severe caries were subjected for plaque collection from intact enamel, white spot lesions, dentine lesions and deep dentine lesions. After DNA extraction, 16S rRNA gene amplified and sequenced to detect bacterial species identities. 50% of the 197 bacterial species/phylotypes were uncultivable, 22 new phylotypes were identified. In white spot lesions, *Actinomyces* and *non-mutans Streptococci* were most abundant, in an advanced stage of the disease, known acid producing species were detected. Also, it has been found that species such as *Atopodium spp., Actinomyces spp., Propionibacterium spp., Bifidobacterium spp., Lactobacilli spp. and Viellonella* plays important role in caries progression in addition to *Streptococcus mutans* (Aas *et al.* 2008).

In Health status, it is important to identify and understand the present commensal microbiota to prevent disease. A study conducted utilising saliva samples from 74 patients in the age range from 3-18 years old to assess microbiota associated with the transition from primary to permanent dentition. 16S rRNA genes were extracted from samples amplified and sequenced using 454 pyrosequencing and further analysed by phylogenetic microarrays. Results showed 8 phyla and 113 higher taxa in saliva. There was an increase in *Bacteriodetes, Viollenellaceae* and *Spirochaetes* with age. In addition, there was an abundant amount of *Proteobacteria* in primary dentition in compare to *Bacteriodetes*, however in contrast to the stage of permanent dentition where there was an equivalent amount of *Bacteriodetes* to *Proteobacteria*. Moreover, it was concluded that *Porphyromonas catoniae* and *Neisseria flavescens* have the potential to be identified as markers of oral health status because of the high signal acquired from probes that targeting these species in caries-free subjects (Crielaard *et al.* 2011).

Chapter 3 Effect of Mineral Trioxide Aggregate (MTA) Setting Time on its Interfacial Properties with Adhesive Materials; an *In Vitro* Study

3.1 Introduction

Biocompatible materials, including setting calcium hydroxide and mineral trioxide aggregate (MTA), have been used widely for vital pulp treatment of symptomatic/asymptomatic teeth. The bond between these pulp protection materials and restorative materials such as glass ionomer cement (GIC) or resin composite (RC) is essential to seal the pulp protected tooth. (Shin *et al.* 2014). Lack of adequate seal may result in the entry of bacteria into the pulp (Paterson 1976, Bergenholtz 2000, Wells *et al.* 2002, Tselnik *et al.* 2004). MTA is a hydrophilic cement composed of calcium oxide, silica and bismuth oxide (Oskoee *et al.* 2011). It has poor mechanical properties that preclude the definitive restoration of the treated tooth in a single visit. MTA has a reported setting time of between 75 minutes and 73 hours (Chng *et al.* 2005). Therefore, it has been used in conjunction with materials including GIC or resin-modified glass ionomer cement (RMGIC) to provide a barrier until maturation (Yesilyurt *et al.* 2009). This requires a two-step clinical procedure to ensure complete setting of MTA (Rada 2013). Light-cured MTA, which contains approximately 45% weight mineral material (type III Portland cement), 10% weight radiopaque component, 5% weight hydrophilic thickening agent (fumed silica) and approximately 45% resin (Suh *et al.* 2008), has a reduced setting time up to 1.7 mm thickness per 20 seconds of light curing, which may avoid the risk of untimely dissolution; however, it is not as osteoconductive as the non-light-cured MTA (Gomes-Filho *et al.* 2008, Gandolfi *et al.* 2012).

Studies have recommended a direct seal in one session visit in cases of indirect pulp protection (Oliveira *et al.* 2006, Maltz *et al.* 2012). The definitive restoration of a tooth directly after minimally invasive partial caries removal, rather than following a sequential stepwise excavation procedure, may result in a higher success rate of the procedure (Bjørndal 2013). Therefore, the long-term synergy between different materials used in one-visit vital therapy is necessary. Studies have shown that etching and bonding procedures before resin composite placement affect adversely the setting reaction of fresh MTA during pulp-capping procedures (Nandini *et al.* 2007, Yesilyurt *et al.* 2009). Therefore, recognising the effect of bonding of resin-based composite or GIC to MTA is relevant clinically in order to predict materials' behaviour in one-visit planned restorations. Determining the bond strength *in vitro* between two materials gives the clinician an initial knowledge about the expected clinical behaviour (Neelakantan *et al.* 2012).

Although *in vitro* tests are not a substitute for clinical studies to assess outcome success of restorations, they are often the favoured method of assessing bond performance as they provide rapid data production and are less demanding to perform (Green and Banerjee 2011). Micro-tensile and shear bond strength tests are the most frequently used tests in adhesive dentistry. The shear bond strength test was chosen for this study in order to obtain a comparison with other similar studies (Tunç *et al.* 2008, Yesilyurt *et al.* 2009, Atabek *et al.* 2012, Neelakantan *et al.* 2012, Shin *et al.* 2014). Understanding the nature of the interface between different restorative materials in laminate restorations provides the evidence-base to achieve the greatest longevity of such restorations. The direct placement of resin composite over the MTA pulp cap provides a simple and reliable operative technique to restore the pulp-capped tooth with a definitive restoration without the need for a temporary/provisional restoration.

3.2 Aims and Hypotheses:

This *in vitro* study aimed to evaluate the shear bond strength of white mineral trioxide aggregate (MTA) and GIC or RC after 10 minutes, 24 hours, 72 hours and 30 days intervals of MTA setting time. It also aims to assess the mode of failure of the interface between the MTA and RC or GIC.

The study tested the null hypotheses that there is no difference in shear bond strength between MTA and resin composite or GIC and also, that the setting time period of MTA had no effect on the shear bond strength of resin composite and GIC and there is no difference between RC and GIC in terms of interfacial failure mode (IFM) to MTA; also there will be no effect of MTA setting time on the IFM of both materials.

3.3 Material and Methods

3.3.1 Sample Preparation

Eighty acrylic resin templates were prepared with 4mm diameter and 3mm depth standardised cavities for the MTA. Plastic cylindrical transparent tubes with dimensions of 3.2mm diameter by 3mm height were prepared to act as moulds for the RC or GIC restorative material. MTA applicap (Acteon Pierre Rolland, Merignac France) was activated and mixed in an amalgamator (Ultramat 2, SDI, Bayswater, Australia) as per manufacturer's instructions. MTA was extruded into the prepared acrylic cavities using a delivery plunger (No. 01/98, 3MESPE) and condensed with an appropriate hand instrument (No. 04/6, Dentsply Ash UK). A cotton pellet or a paper point was used to remove the extra moisture to prevent overhydration as per manufacturer instructions. Four groups of 20 MTA samples each were left to set for 10 minutes, 24 hours, 72 hours and 30 days, respectively, in 37^{o}C and 100% humidity conditions, before bonding to either GIC (Fuji IX GC Corporation, Tokyo, Japan) or RC (N'Durance, Septodont, Cedex, France). Materials used in this study are summarised in Table 3-1.

Table 3-1: Chemical composition of materials used in this study.

Material	Manufacturer	Material composition	Lot Number
White mineral trioxide aggregate	MTA cap, Acteon, Pierre Rolland, Merignac France	Powder: mixing of mineral oxides based on calcium and tungstate. Liquid: water	
GIC	Fuji IXTM GP, GC Corporation, Tokyo, Japan	Powder: fluoro-alumino-silicate glass, polyacrylic acid powder Liquid: polyacrylic acid, Polybasic carboxylic acid	1310171
Resin composite	N'Durance®, Septodont, Cedex, France	The resin-based matrix contains approximately 19 wt % of ethoxylated BisGMA, UDMA and the new dicarbamate dimethacrylate dimer acid. The filler system contains approx. 80 wt % (65 vol %) silanated 40 nm ytterbium fluoride, silanated 500 nm barium glass and 10 nm silica. There is approximately 1 wt % of catalyst, inhibitors and pigments.	100713A
Etch and rinse adhesive	XP bond, DENTSPLY DeTrey GmbH, Konstanz, Germany	Carboxylic acid modified dimethacrylate (TCB resin) Phosphoric acid modified acrylate resin (PENTA) Urethane dimethacrylate (UDMA) Triethyleneglycol dimethacrylate (TEGDMA)	1312000368

		-hydroxyethylmethacrylate (HEMA) Butylated benzenediol (stabilizer) Ethyl-4 dimethylaminobenzoate Camphorquinone Functionalised amorphous silica t-butanol	
Etchant	Etch Gel, HENRY SCHEIN, NY, USA	40% phosphoric acid	K2311-5

In the four RC experimental groups (1, 3, 5 and 7) after 10 minutes, 24 hours, 72 hours and 30 days, respectively (n=10), the set MTA samples were etched with 37% phosphoric acid gel (Etch Gel, HENRY SCHEIN, NY, USA) for 15s, followed by 30 seconds of water rinsing, and 5 seconds of air drying. A type 2 adhesive (etch and rinse) (XP Bond, DENTSPLY DeTrey GmbH, Konstanz, Germany) was applied to the surface of the MTA with a clean micro-brush. Two layers of adhesive were applied before a mild air flow was applied for 5 seconds to evaporate the solvent. The adhesive was light-cured for 20 seconds (Optilux 501, SDS Kerr Sybron, USA) with an output wavelength range of 400-505 nm and output light intensity of 850 mW/cm^2. The transparent plastic moulds were filled with resin composite (shade A2) and were placed on the surface of MTA samples. The composite was condensed and light-cured for 40 seconds from the top plus 40 seconds from the sides under average finger pressure. A scalpel blade was used to cut through and remove the plastic tubes after polymerisation of RC. The specimens were stored at 37^{o}C and 100% humidity for 24 hours before testing. A schematic diagram in Figure 3-1 shows the process of preparation of the specimens and the shear bond strength testing.

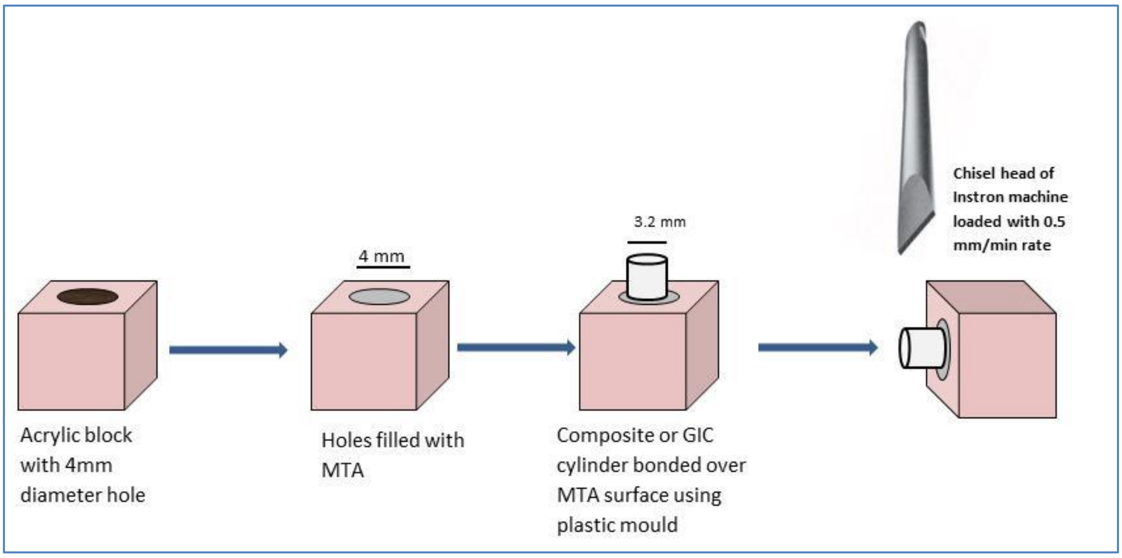


Figure 3-1: Schematic diagram illustrating sample preparation and shear bond strength testing.

In the other four experimental groups of MTA that were bonded with GIC (groups 2, 4, 6 and 8: n=10 / group), the GIC powder and liquid (shade A2) were mixed on a mixing pad with a plastic spatula as per manufacturer's instructions, and placed over the surface of MTA by filling the material into the cylindrical plastic tube over the centre of the MTA's surface. The specimens were allowed to set for 10 minutes within the plastic tubes under average finger pressure to ensure completion of the initial setting reaction of the GIC. The plastic tubes were removed with a sharp scalpel as previously described. Samples were stored at 37^{o}C and 100% humidity for 24 hours. All samples were prepared by the same operator. A flow chart of the experiment groups is shown in Figure 3-2.

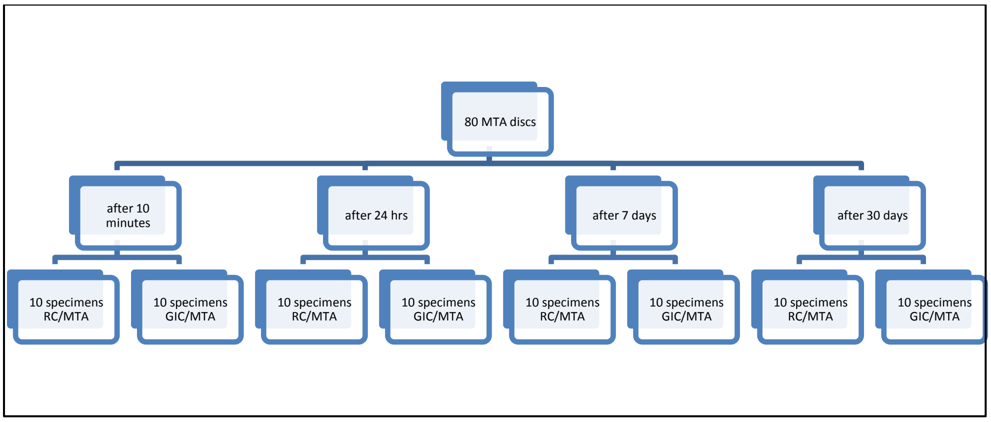


Figure 3-2: Flow chart of experiment groups.

3.3.2 Shear Bond Strength Testing (SBS)

Each specimen was mounted in a universal testing machine (Model 5569 A, Instron, High Wycombe, UK). Bond failure between MTA and resin composite or GIC in each specimen was obtained by using a knife edge blade applied with a crosshead speed of 0.5 mm/min. The values were recorded in N and transformed to MPa by dividing the peak load at failure by the interfacial surface area of the GIC / resin composite and MTA. The failure mode between MTA and resin composite / GIC was assessed optically using a stereomicroscope (Meiji Techno UK, Limited) under magnification 4.5X. All samples were analysed with a stereomicroscope in both parts: the MTA surface and the RC or GIC part.

The modes of failure were categorised into the following categories:

a- Adhesive failure between MTA and restoration.

b- Cohesive failure within MTA.

c- Cohesive failure within the restorative material.

d- Cohesive failure within adhesive in groups 1, 3, 5 and 7.

e- Adhesive failure between adhesive and MTA in groups 1, 3, 5 and 7.

3.3.3 Scanning Electron Microscopy

A scanning electron microscope (FEI, Cambridge, UK) was used to examine the ultrastructure of the interface surfaces (accelerating voltage of 3.5 and 10 kV, working distance of 10 mm, magnifications were at 500x and 2500x). Three randomly selected samples per experimental group were examined and gold sputter-coated before SEM analysis (Emitech K550, UK); two samples were used to scan the interface between MTA and resin composite or GIC longitudinally, and one sample was used to scan part of the interface surface horizontally.

3.3.4 Statistical Analysis

The means and standard deviations of shear bond strength for all groups were calculated. The means of shear bond strengths of all groups were compared using one-way analysis of variance (ANOVA), significance level estimated as $p < 0.05$. Tukey HSD test was used to compare between different groups of RC and GIC. Two-tailed independent sample z test for proportions was used to analyse results of the modes of failure. Statistical analysis was conducted using IBM SPSS statistics version 20 software.

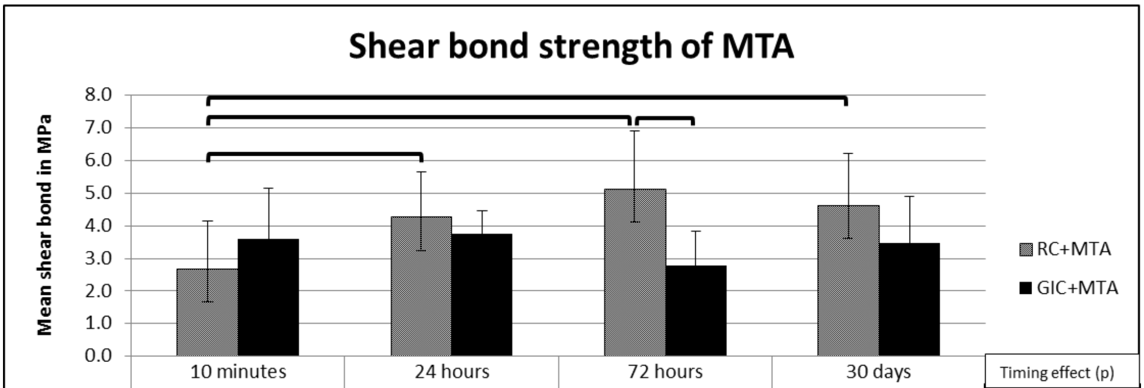
3.4 Results

3.4.1 Shear Bond Strength

The mean shear bond strength (SBS) of each group was calculated and the highest mean SBS was in MTA+RC/72hrs group (5.09 ± 1.79 Mpa) and the lowest mean SBS (2.7 ± 1.3 Mpa) was in MTA+RC/10 minutes group among all groups. The highest mean SBS of MTA+GIC was in the 24hrs group (3.74 ± 0.70 Mpa). There were no pre-test failures in any of the experimental groups. Analysis of variance (ANOVA) used for comparison between all experimental groups revealed the presence of highly significant differences between all groups ($p=0.002$).

Material effect: Mean SBS of RC/MTA was higher than mean SBS between GIC+MTA in the 24 hrs, 72 hrs and 30 days groups, respectively, except in the 10 minutes group, where the mean SBS of GIC/MTA was higher than RC/MTA. However, there was no significant difference in SBS between RC and GIC at any time point of MTA setting time ($p>0.05$), except at 72 hrs, where the SBS of RC/MTA was significantly higher than the SBS of GIC/MTA.

Timing effect: Results between individual groups of RC using Tukey HSD test show significant differences ($p=0.045$, $p=0.002$, and $p=0.015$) between the 10 minutes and 24 hrs, 72 hrs and 30 days groups, respectively. There was no significant difference ($p>0.05$) between 24 hrs and 72 hrs and 30 days, respectively. Also, there was no significant difference between the 72 hrs and 30 days groups ($p>0.05$). In contrast, results between individual groups of GIC+MTA using Tukey HSD test showed no significant difference between any of the GIC groups ($p>0.05$). Using ANOVA, there was a statistically significant effect of timing of MTA setting (10 min, 24 hrs, 72 hrs and 30 days) on the SBS of MTA and RC ($p=0.008$), and there was no statistically significant effect of time on the SBS of MTA and GIC ($p=0.3$) as shown in Figure 3-3.



	10 minutes	24 hours	72 hours	30 days	Timing effect (p)
RC+MTA	2.7 ± 1.3	4.2 ± 1.4	5.1 ± 1.7	4.6 ± 1.6	0.008
GIC+MTA	3.6 ± 1.5	3.7 ± 0.7	2.8 ± 1.0	3.5 ± 1.4	0.300

Figure 3-3: Mean SBS (±standard deviation), Tukey HSD post-hoc and ANOVA results of all experimental groups. Crossbars indicate statistically significant differences between groups ($P < 0.05$).

3.4.2 Modes of Failure

Assessment of modes of failure revealed combined cohesive and adhesive failures in some specimens at the same time and each one categorised separately. High proportions of cohesive failures within the substrate material (MTA) were found in all groups (category b) and there was no significant difference in cohesive failures of MTA between groups of RC or GIC or between different times ($p>0.05$). Conversely, there was no cohesive failure in RC (category c) observed within RC groups and very low cohesive failure in GIC (category c) in the GIC/MTA 24 hrs group (20%), with no significant difference between RC and GIC ($z=1.49$, $p=0.13$). However, there was significantly higher cohesive failure within the bonding agent (category d) of RC groups in 72 hrs (60%) compared to 10 min. (0%), 24 hrs (0%) and 30 days (0%) ($z=2.29$, $p=0.003$, respectively).

All groups in RC or GIC exhibited variable numbers of adhesive failures, either between MTA and bonding agent or between MTA and GIC at different points of time. In the RC groups, adhesive failures were 90% in both the 10 min and 24 hrs groups, decreased later to 30% and 10% in the 72 hrs and 30 days groups, respectively. There was a significant difference in adhesive failures between the 10 min and 72 hrs (z=3.57, p=0.0003) and 30 days (z=2.73, p=0.006) groups respectively. In the GIC groups, there were 70%, 80%, 90% and 60% adhesive failures to MTA in 10 min, 24 hrs, 72 hrs and 30 days, respectively, with no significant difference between groups ($p>0.05$). Results of modes of failures assessment are shown in Table 3-2. Representative stereoscopic images of samples of the modes of failures are shown in Figure 3-4.

Table 3-2: Distribution of modes of failure and percentages recorded in all experimental groups.

Modes of Failures in RC/MTA	10 MINUTES	24 HOURS	72 DAYS	30 DAYS	trend
b= Cohesive within MTA	60%	90%	90%	90%	
d= Cohesive within Bonding agent	0%	0%	60%	0%	
e= Adhesive between bonding agent/MTA	90%	90%	30%	10%	
c= Cohesive within RC	0%	0%	0%	0%	
Modes of Failures in GIC/MTA	**10 MINUTES**	**24 HOURS**	**72 DAYS**	**30 DAYS**	**trend**
a= Adhesive between MTA and GIC	70%	80%	90%	60%	
b= Cohesive within MTA	60%	70%	70%	90%	
c= Cohesive within GIC	0%	20%	0%	0%	

MTA, mineral trioxide aggregate; RC, resin composite; GIC, glass ionomer cement

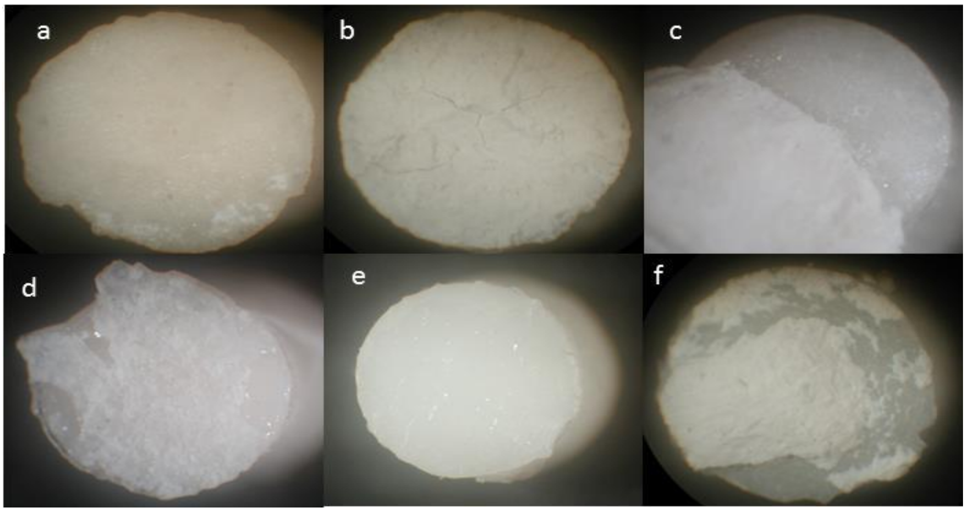


Figure 3-4: Representing stereomicroscopic images of modes of failure; (a) adhesive failure MTA/GIC, (b) cohesive failure in MTA, (c) cohesive failure in GIC, (d) cohesive failure in bonding agent, (e) adhesive failure in bonding agent, (f) mixed cohesive/adhesive

3.4.3 Scanning Electron Microscopy

The scanning electron microscopic analysis of the interface between MTA and GIC showed vertical and horizontal cracks in GIC; these were limited to the superficial interfacial layer of GIC with MTA, as shown in Figure 3-5.

Resin composite-MTA interface analysis revealed a continuous interface with fewer gaps as shown in Figure 3-6 and a filamentous network of adhesive infiltrating the void spaces between the MTA particles forming a mesh-like structure after adhesive failure as shown in Figure 3-7.

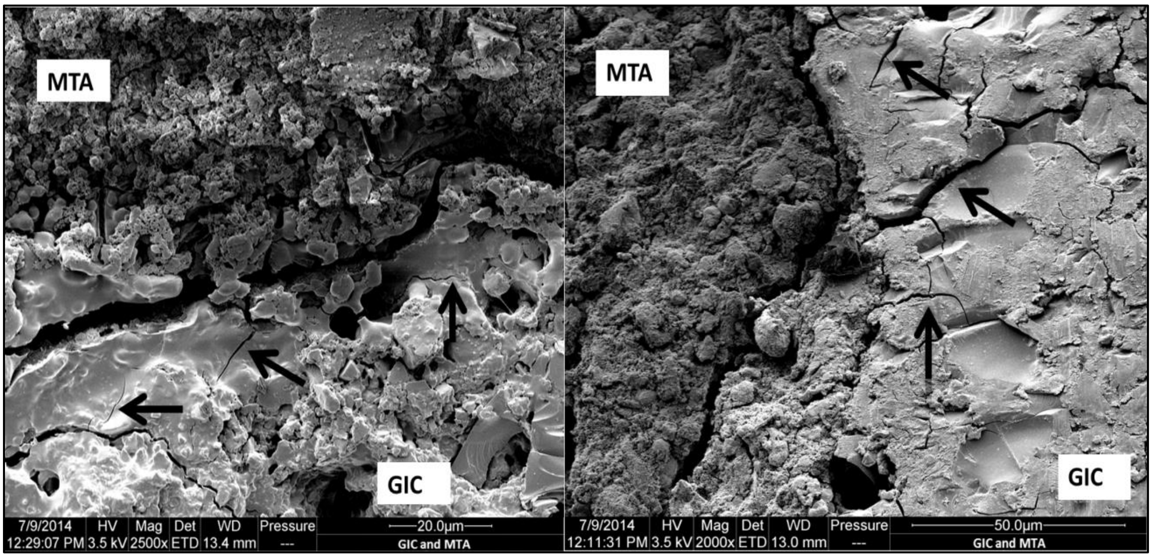


Figure 3-5: Longitudinal section SEM images of the interface between GIC and MTA (3.5 kV, ×2,000 and ×2,500). Arrows show the cracks in the GIC layer.

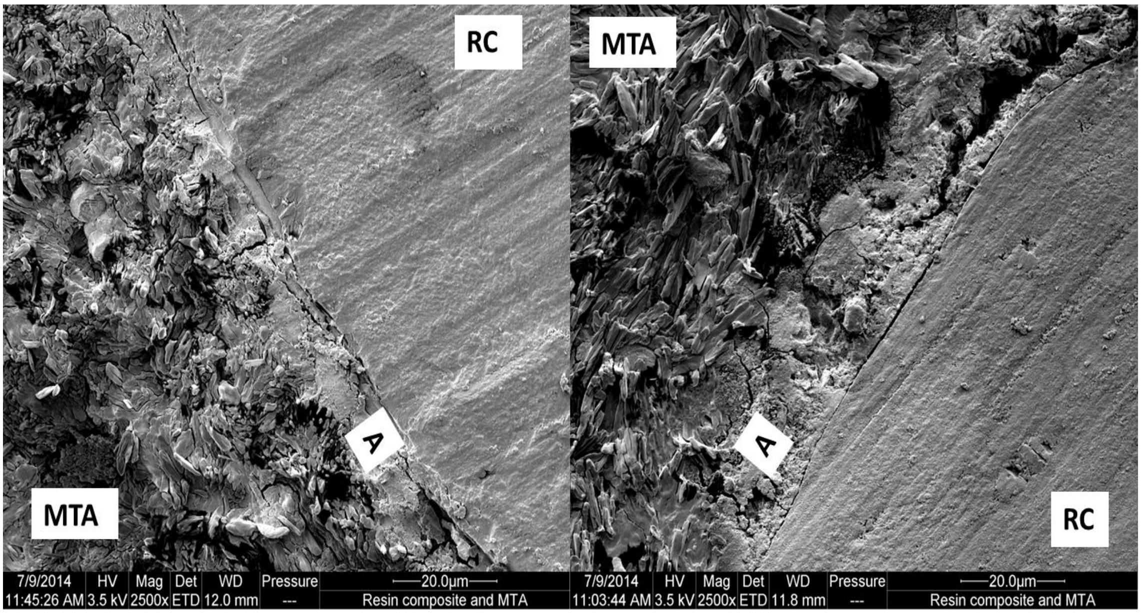


Figure 3-6: Longitudinal section SEM images of the interface between RC, adhesive (A) and MTA (3.5 kV, ×2,500).

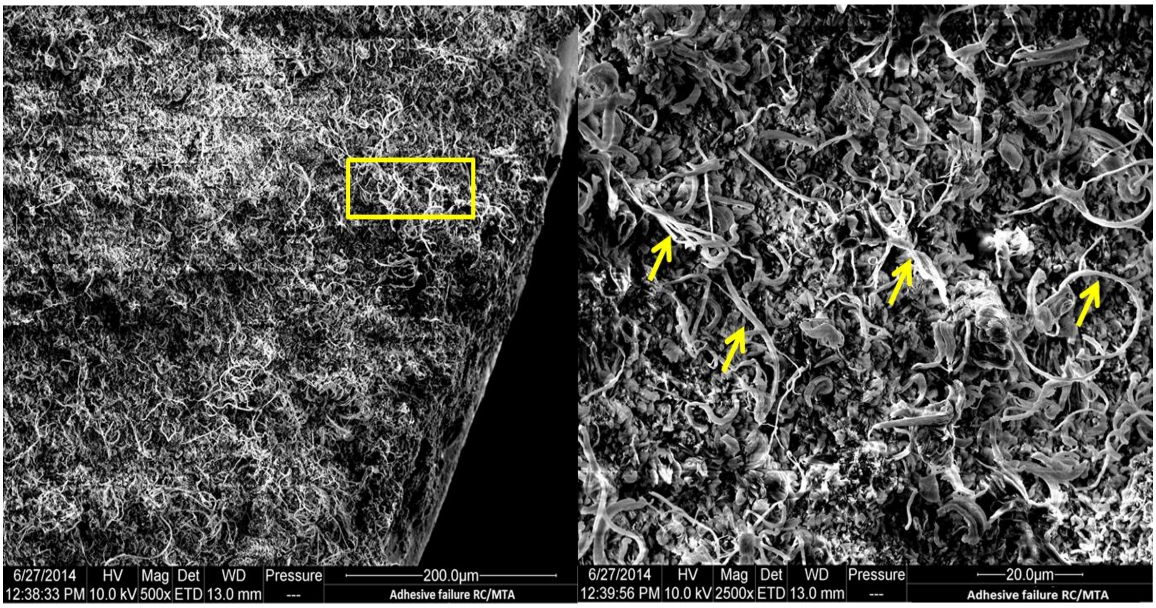


Figure 3-7: Surface SEM images of a resin composite /adhesive sample after adhesive failure. Arrows show filaments of bonding agent resin infiltrated into MTA spaces after adhesive failure, with 10.0 kV, 500x and 2500x magnification.

3.5 Discussion

Definitive restoration of cavities immediately after pulp protection, with resin composite, is a frequent occurrence especially in one-visit sessions (Maltz and Alves 2013). GIC may be used as a definitive restoration in case of the absence of adequate enamel for resin composite bonding. In such restorations, the bond between the pulp-capping agents and resin composite or GIC is critical in the overall treatment success (Paterson 1976).

Adhesive materials like RC provide a better seal than GIC in coronal restorations. An adequate bonding of RCs to pulp-capping biomaterials produced an even distribution of stresses over the adhesive layer between the two materials (Oskoee *et al.* 2011).

The most common method to evaluate adhesive properties of restorative materials is bond strength assessment. The micro-tensile and shear bond strength tests are the most frequently used tests in adhesive dentistry. However, considering that MTA is a brittle material, it is not possible to subject it to micro-tensile testing as it would not survive the procedure. Hence, the shear bond strength test was chosen for this study similar to other studies that used MTA as a substrate material (Tunç *et al.* 2008, Bayrak *et al.* 2009, Neelakantan *et al.* 2012, Atabek *et al.* 2012, Ajami *et al.* 2013, Shin *et al.* 2014). Two factors affected shear bond strength values: the type of the material used and the timing of setting of MTA before bonding. The results showed a multifactorial effect of both factors on the shear bond strength in all groups.

Some studies have suggested using RC directly over partially set MTA, while others have proposed layering the freshly mixed MTA with GIC after waiting 45 minutes (Ballal *et al.* 2008, Neelakantan *et al.* 2012). In this study, manufacturer instructions of MTA suggest to wait 5-6 minutes before filling placement. Ten minutes was chosen to have better hardening of MTA. The bond of RC to partially set MTA was weaker than that of GIC to partially set MTA, however, it was higher in later setting time groups. Also, there was no statistically significant effect of MTA setting time on the SBS to GIC, whereas there was a statistically significant effect of MTA setting time on SBS to RC (Figure 3-3). Therefore, layering of MTA with RC is time-dependent and non-time-dependent in the case of layering with GIC. This concurs with the findings that time of GIC placement does not affect the setting reaction of MTA (Eid *et al.* 2012).

GICs adhere to dental hard tissues via a physicochemical process involving an ionic exchange between the interfacial substrates. The phosphate ions in the tooth structure are replaced by carboxyl groups of GIC and the latter form ionic bonds with calcium ions of dental hydroxyapatite (Glasspoole *et al.* 2002). The shear bond strengths of conventional GICs to enamel and dentine or metals are relatively low, ranging between 3 and 7 MPa (Burgess *et al.* 1994, Erickson and Glasspoole 1994). Due to the higher percentage of hydroxyapatite in enamel, the bonding to enamel is likely to be stronger than the bonding to dentine. In the context of the present study, the SBS of restorative materials to MTA ranged between 2 and 5 MPa. The possible interfacial reactions between GIC and MTA include the carboxyl group of the polyacrylic acid interacting with the calcium of the MTA to form calcium salt complexes; the formation of calcium salts in the interface was not affected by the MTA setting time intervals (Nandini *et al.* 2007). Alternatively, a by-product formed by the condensation of silicate hydrate of MTA with that of GIC creates a chemical bond (Nandini *et al.* 2007) in addition to the porous surface of MTA, which may enhance the adhesion of GIC to MTA by micromechanical means. However, MTA/GIC reaction was affected by the condition of moisture (dry or wet setting condition) (Eid *et al.* 2012).

The mean SBS of resin composite to MTA was higher than the mean SBS of GIC to MTA except in the early setting groups (10 minutes). Therefore the first null hypothesis was rejected. The higher bond strength to resin composite may be due to the fact that the phosphoric acid etch provides a clean surface with a honeycomb pattern, increasing the micromechanical attachment, and hence the higher bond strengths observed. These results are in agreement with Ajami *et al*. (Ajami *et al.* 2013). It has been reported that the surface gel-like amorphous structures and needle-like crystals are removed (eroded) during acid etching of MTA (Kayahan *et al.* 2009, Oskoee *et al.* 2011, Shin *et al.* 2014). This selective removal of matrix from the periphery of crystals without a significant loss of MTA structure can produce an ideal surface for bonding with resin composite (Kayahan *et al.* 2009, Shin *et al.* 2014). However, Oskoee and colleagues established that surface etching of MTA is not necessary prior to resin composite restoration using a total-etch adhesive system (Oskoee *et al.* 2011). In this study, the resin composite was bonded with a total-etch adhesive system rather than a type 3 or 4 self-etch adhesive, because the findings from previous studies have demonstrated that total-etching adhesives performed better than one-step self-etching adhesives when used to bond resin composite or compomer to MTA (Tunç *et al.* 2008, Bayrak *et al.* 2009, Atabek *et al.* 2012).

It is clear from the results of the present study that RC produces higher shear bond strengths than GIC when bonded to MTA, particularly after 72 hours' setting time. The lower SBS of RC to MTA compared to that of GIC in the 10 minutes setting groups may be attributed to the fact that the water used to rinse the phosphoric acid etchant before application of the adhesive could have altered the microstructure of the MTA (Bodanezi *et al.* 2008). This result coincides with those of a study which found that acid-etch procedures affected the compressive strength and surface microhardness of MTA and thus advised postponing acid etching of MTA until 96 hours after MTA mixing to permit the material to reach its ideal physical properties (Kayahan *et al.* 2009, Atabek *et al.* 2012). However, these results are not in agreement with those of Neelakantan *et al* (2012), who found higher SBS of RC when bonded immediately to MTA compared to 45 minutes and 24 hours intervals. Their explanation was that the MTA exhibits greater porosity at the initial stage and they suggest that the adhesive could penetrate more deeply in MTA, leading to stronger bond at the initial stage than a late stage. However, it is clear that the poor mechanical properties of MTA in the initial setting phase have a high impact on the low bond strength to RC.

The second null hypothesis was rejected because there was a statistically significant effect of MTA setting times on the SBS of MTA with resin composite ($p=0.008$). In contrast, there was no statistically significant effect of MTA setting timing on SBS of

MTA and GIC ($p>0.05$). This is in agreement with the findings of other studies (Yesilyurt *et al.* 2009, Atabek *et al.* 2012), where investigators found similar mean shear bond strengths between GIC and MTA after 45 minutes' and 72 hours' setting time of MTA. This is attributed to the fact that major interaction between GIC and MTA is a chemical interaction with little effect of micromechanical interlocking. In contrast, most of the interactions between RC and MTA depend on micromechanical retention, which was positively proportional to the degree of MTA hardening with time.

During MTA preparation, different liquids can affect its physical properties and set time (Roberts *et al.* 2008). The critical role of water in the setting of MTA has previously been shown (Camilleri *et al.* 2005), therefore it is believed that there is a detrimental effect of placing GIC over non-setting MTA, in that GIC will absorb water and lead to incomplete hydration and increase the porosity of MTA (Camilleri 2011). However, other studies concluded that the effect of GIC placement over MTA after different time intervals and setting conditions was transient; also, the GIC and deeper layers of MTA did not seem to be affected (Ballal *et al.* 2008, Eid *et al.* 2012). And because the interaction may be by the formation of calcium salts, which is similar to the interaction of GIC with the tooth structure, it is assumed that the setting of GIC is not hindered by combining with MTA at different time intervals (Nandini *et al.* 2007). It was observed in SEM images in this study that there were micro-cracks associated with the GIC layer; this observation was similar to that reported previously which may be caused by vacuum drying of the SEM (Camilleri 2011).

In the present study, the most common mode of failure was the cohesive one within the MTA substrate, which indicates a stronger chemical/mechanical bond at the interface. On the other hand, this finding may reveal weak mechanical properties of the MTA substrate material. However, the third null hypothesis was rejected as there was a notable constant rate of the adhesive failure between GIC and MTA with time, in contrast to the significant decrease in RC trend in adhesive failures by time (Table 3-2). The failure mode results coincide with the results of SBS, as the bond between resin composite and MTA increase when more time is given to MTA to set. However, further studies are required firstly to assess the effect of RC on the setting interactions of MTA, and secondly to assess the restorative clinical success of vital pulp therapy using direct RC restoration over the MTA capped pulps.

One of the limitations of the present study was the use of the shear bond strength test, which may not simulate fully the various natural stresses on substrate materials in teeth treated with vital pulp therapy. Natural, synthetic saliva or phosphate buffered saline was not used for storing the samples which may influence the bond and seal between different materials of the study. However, in actual clinical situations, MTA might be exposed to distilled water or saline from the coronal side as a means of moisture supply for the setting reaction (Han *et al.* 2010). Clinical studies will help to assess the relative success and outcome of any bond between two materials.

3.5.1 Conclusions:

Shear bond strengths between RC and fully set MTA were significantly higher than those of GIC to fully set MTA after 24 hours, 72 hours and 30 days of MTA mixing; however, they were lower compared to those of GIC to partially set MTA. A high adhesive failures proportion was observed between partially set MTA and RC compared to that between fully set MTA and RC, which decreased significantly with time compared to that between GIC and MTA which was non-time-dependent.

3.5.2 Clinical Significance

Direct placement of RC over partially set MTA might result in a weak mechanical/chemical bond between them in a single-visit restoration of teeth capped with MTA.

Chapter 4 Remineralisation of Natural Caries-affected Dentine after Mechanical Rotary or Chemomechanical Caries Excavation: An *In Vitro* Study

4.1 Introduction:

Traditionally, in order to provide a sound mineralised structural base for the restoration and to arrest further cariogenic activity within the lesion, removal of all carious dentine (infected and affected) was advised. However, the predictability of such a procedure is questionable as regards the increased risk of pulp exposure and devitalisation (Van Thompson *et al.* 2008). Therefore, the preservation of pulp health and hard tissues is recommended when managing deep caries lesions (Schwendicke *et al.* 2016). Minimally invasive selective removal of carious tissue is the treatment of choice in deep cavitated dentine lesions in teeth with sensible / asymptomatic pulps (Schwendicke *et al.* 2016), in reducing the risk of pulp exposure (Bjørndal 2011, Ricketts *et al.* 2013), preserving hard tissues (caries-affected dentine (CAD)) and arresting the cariogenic activity of bacteria (Banerjee *et al.* 2000b, Lula *et al.* 2009). Although concerns have been raised regarding lower fracture strength and increased microleakage associated with partially excavated teeth compared to completely excavated teeth (Hevinga *et al.* 2010), the annual failure rate for teeth with selective caries removal was reported to be similar or better to those with complete excavation. The latter group experienced most failures due to pulpitis and abscess formation (Schwendicke *et al.* 2013b). Radiographic observations of the demineralised area under the restoration after selective caries removal showed an increased radiodensity at follow-up periods (Maltz *et al.* 2002, Alves *et al.* 2010). Remineralisation of the remaining demineralised dentine after selective caries removal is a prerequisite to prevent further pulp irritation and to enhance mechanical integrity of the tooth-restoration complex.

Remineralisation of caries-affected dentine can be achieved using bioactive materials such as calcium hydroxide, mineral trioxide aggregate (MTA) or Biodentine™. It is difficult clinically to differentiate CAD due to the non-selective nature of the traditional carious tissue excavation techniques. Chemomechanical caries removal systems such as Carisolv™ gel (Rubicon Lifesciences, Sweden) prevent excessive removal of hard tissues by providing a self-limiting end point for caries excavation (Banerjee *et al.* 2000b). The effect of Carisolv™ gel on the chemical, morphological, bacteriologic and mechanical characteristics of the retained dentine surface have been investigated (Banerjee *et al.* 2000b, Lager *et al.* 2003, Sakoolnamarka *et al.* 2005, Hamama *et al.* 2013, P. M. Viral *et al.* 2013, Ramamoorthi *et al.* 2013, de Almeida *et al.* 2013). Results revealed a significant effect of the type of carious tissue excavation methods on the Vickers hardness of dentine, where Carisolv™ gel resulted in lower VHN compared to rotary bur excavation (Sakoolnamarka *et al.* 2005, Mollica *et al.* 2012, Hamama *et al.* 2013). Varied patterns of dentine surface texture resulted from different excavation methods. Carisolv™ gel resulted in no obvious smear layer and open dentine tubules compared to prominent smear layer and occluded dentine tubules with carbon-steel bur excavation (Banerjee *et al.* 2000a, Hamama *et al.* 2013). Carisolv™ gel excavation retains 50 μm more carious dentine than excavation with rotary burs (Splieth *et al.* 2001) and it is effective in terms of the extent of carious dentine removed relative to the autofluorescence signature of the remaining dentine (Banerjee *et al.* 2000b).

Remineralisation of this demineralised CAD layer is of clinical importance in maintaining the pulp health and the mechanical integrity of the tooth-restoration complex, which is controlled typically by the rate of ions exchange in this area (Kawasaki *et al.* 2000). Supplementation and deposition of calcium and phosphate ions into demineralised dentine result in net mineral gain (Cochrane *et al.* 2010).

MTA as a calcium source is a bioactive material forming apatite when it comes in contact with phosphate-containing fluids (Bozeman *et al.* 2006, Tay *et al.* 2007). *In vitro* MTA / phosphate-containing fluid systems have been used to initiate apatite deposition in demineralised dentine with promising results (Ten Cate 2001, Reyes-Carmona *et al.* 2009, Reyes-Carmona *et al.* 2010, Qi *et al.* 2012). Most of these *in vitro* remineralisation studies utilise artificially demineralised dentine samples. Clinically, it is important to explore the rate of remineralisation of natural caries-affected dentine in the presence of calcium and phosphate ions sources after excavation with Carisolv™ gel or rotary burs.

Raman spectroscopy has been used previously to characterise the different layers of sound and carious dentine lesions and to detect demineralisation / remineralisation in enamel / dentine (Koutsopoulos 2002, Almahdy *et al.* 2012, Milly *et al.* 2014). It is both a qualitative and quantitative method for monitoring chemical composition in natural and synthetic materials (Tsuda and Arends 1997). It relies on inelastic scattering, or Raman scattering, of monochromatic light, usually from a laser in the visible, near infrared, or near ultraviolet range. The laser light interacts with molecular vibrations, phonons or other excitations in the system, resulting in the energy of the laser photons being shifted up or down. The detected shift in energy of photon gives information about the vibrational modes in the system. Infrared spectroscopy yields similar, but complementary information.

A reduction in surface microhardness indicates a dissolution, degradation or demineralisation in hard tissue structures (Salem-Milani *et al.* 2015). Raman spectroscopy and Knoop microhardness were used conjointly in this study to monitor the remineralisation in dentine.

4.2 Aims

This study aimed to investigate and monitor the potential change in mineral content and microhardness of natural CAD after excavation with Carisolv^{TM} gel or rotary burs, using or not an ion source.

4.3 Null Hypotheses

The first null hypothesis was that the mineral level in dentine excavated with Carisolv^{TM} gel / rotary bur will be equal to each other and to that of sound dentine after 14 days' storage. The second null hypothesis is that the microhardness of dentine excavated with Carisolv^{TM} gel / rotary bur will be equal to each other and to that of the sound dentine after 14 days' storage. The third hypothesis was that there is no difference in remineralisation potential of CAD after storage either in distilled water (DW) or simulated body fluid (SBF).

4.4 Materials and Methods

4.4.1 Samples Preparation

4.4.1.1 Teeth Sectioning

Twenty human extracted carious molars and five sound teeth were collected after obtaining the informed consent of the patients after NRES Committee London-Riverside ethical approval (14/LO/0123). Teeth were stored in physiologic saline at 4^{o}C for no more than one month and were imaged radiographically to verify the depth of the carious lesion. Teeth with carious lesions extending from enamel into the middle third of dentine were selected. Each carious tooth was cleaned with tap water for two minutes and then sectioned mesiodistally using a water-cooled diamond-impregnated circular saw (XL 12205, Benetec Ltd., London, UK) (Figure 4-1).

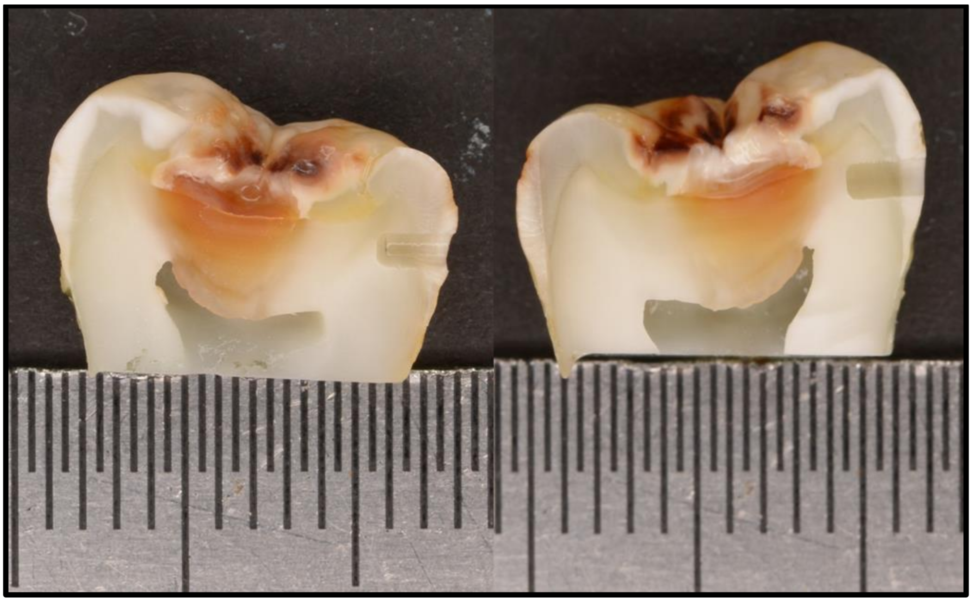


Figure 4-1: Sectioning of the carious tooth into two halves, each half excavated with either Carisolv™ or rotary burs.

Teeth were divided into two groups of 20 halves; the first stored in DW and the second in SBF, both for 14 days. Each group was further divided into two further subgroups (n=10 hemisections per subgroup): one excavated with Carisolv™ gel / hand instruments (CDW, CSBF) and the other excavated with rotary carbon-steel burs in a slow-speed handpiece (RDW, RSBF). The five sound posterior teeth were sectioned mesiodistally and used as control subgroups (SDW, SSBF) (n=5 halves / subgroup) and also stored either in DW or SBF. Points of measurements for mineral contents and Knoop microhardness on sound controls were dete

4.4.1.2 Mechanical Rotary Burs

After gaining appropriate access with a tungsten carbide bur ref. 878-2800 (Henry Schein, UK) in a high-speed air turbine handpiece Alegra TE-98 (W&H Dentalwerk Bürmoos GmbH, Salzburg, Austria), carbon-steel round burs (Ash Instruments, Dentsply, Gloucester, UK) in a slow-speed handpiece WA56A (W&H Dentalwerk Bürmoos GmbH, Salzburg, Austria) (sizes 4 and 5; 5000-10000 rpm) were used to remove carious dentine with circular light brush strokes. A sharp dental explorer was used to verify the removal of soft 'caries-infected' dentine, attempting to retain CAD which is sticky-scratchy in texture (clinical criteria) (Banerjee and Watson 2015, Schwendicke *et al.* 2016).

4.4.1.3 Carisolv™ System

The other half was excavated using chemo-mechanical Carisolv™ gel (RLS Global AB, Gothenburg, Sweden, batch no. E-1041-1) (Figure 4-2), supplied as a twin-syringe, two-component system. The gel was auto-mixed in the correct proportions (Static Mixer, RLS Global AB, Gothenburg, Sweden) prior to application on the carious lesion. Drops of gel were applied on the lesion after gaining the appropriate access with hand or rotary instruments at the level of the enamel-dentine junction. After 30 seconds, Carisolv™ hand instruments of the appropriate size (1-6) were used to abrade away soft caries. After the gel became cloudy, the cavity was rinsed with water and the procedure repeated until the gel remained clear; this helped verify the excavation endpoint.

Figure 4-2: Carisolv™ gel is a twin-syringe system providing *in situ* mixing of sodium hypochlorite in one syringe and three amino acids (lysine, leucine and glutamic acid) in a methylcellulose gel preparation in the other syringe.

4.4.1.4 MTA Application and Restoration

MTA caps (Acteon, Pierre Rolland, Merignac, France) containing mineral trioxide aggregate powder and distilled water were activated as per manufacturer instructions. MTA was applied on the base of the prepared cavities and condensed with appropriate instruments. In order to prevent cement leakage across the cut surface, a glass slide was applied tightly against the sectioned tooth surface. The extra moisture was removed with a cotton pellet or a paper point. MTA was allowed to set for one hour in 100% humidity at 37°C to allow the initial setting of MTA before applying the etching gel on the remaining cavity surfaces followed by rinsing with water and air-drying. A layer of Scotchbond™ Universal dental adhesive (3M Oral Care, USA) was applied and light cured for 20 seconds before placing a resin composite (N'Durance, Septodont, Cedex, France) restoration to prevent dislodgment of the cement during the storage period. After storage for 24 h at 37°C either in DW or SBF, the sectioned surfaces were polished using 1200, 2000, 2500 and 4000 grit carborundum papers for one minute each and cleaned in an ultrasonic bath with deionised water for three minutes after each polishing step, before a final cleaning in an ultrasonic bath for five minutes (Watson *et al.* 2014). A scalpel blade was used to create reference points perpendicular to the tooth / restoration interface under magnification to enhance reproduction of Raman and micro-hardness recordings at one and 14 days after immersion in the chosen solution. Sample restoration is shown in Figure 4-3.

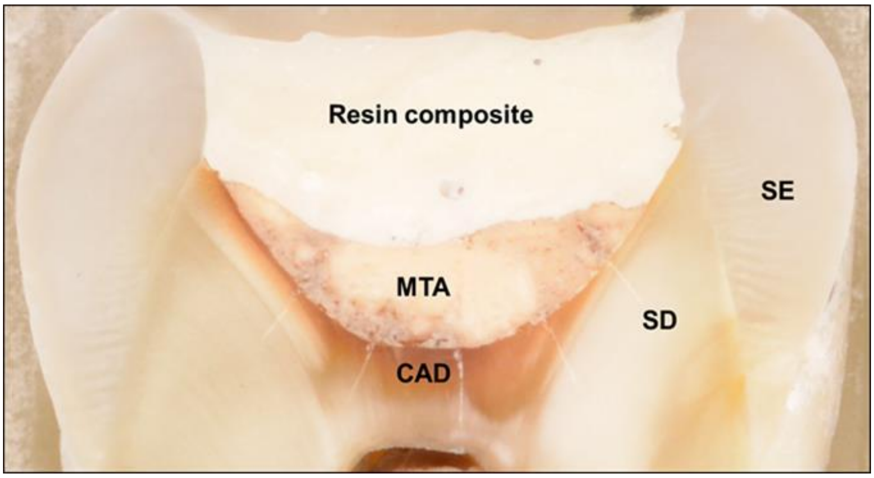


Figure 4-3: MTA applied on the base of the prepared cavities in group 1 and 2 followed by resin composite. CAD: caries-affected dentine, SE: sound enamel, SD: sound dentine.

4.4.1.5 Remineralisation Protocol

Simulated body fluid (SBF) was prepared by dissolving 136.8 mM NaCl, 4.2 mM $NaHCO_3$, 3 mM KCl, 1 mM $K_2HPO_4.3H_2O$, 2.5 mM $CaCl_2$, and 0.5 mM $NaSO_4$ in deionised water, adding 3.08 mM sodium azide to prevent bacterial growth. The pH of the solution was controlled by alternate adding of 0.1 mM HCl and 0.1 mM Tris base until attaining a pH of 7.4. Each tooth half was stored in a glass scintillation vial filled with 15 mL of SBF or DW for a period of 14 days. Evaporation of the solution was prevented by capping of the glass vials and stored in an incubator at 37^oC. The remineralisation medium was changed every three days, with its pH monitored weekly (Kim *et al.* 2010). Groups and subgroups of the study illustrated in Figure 4-4

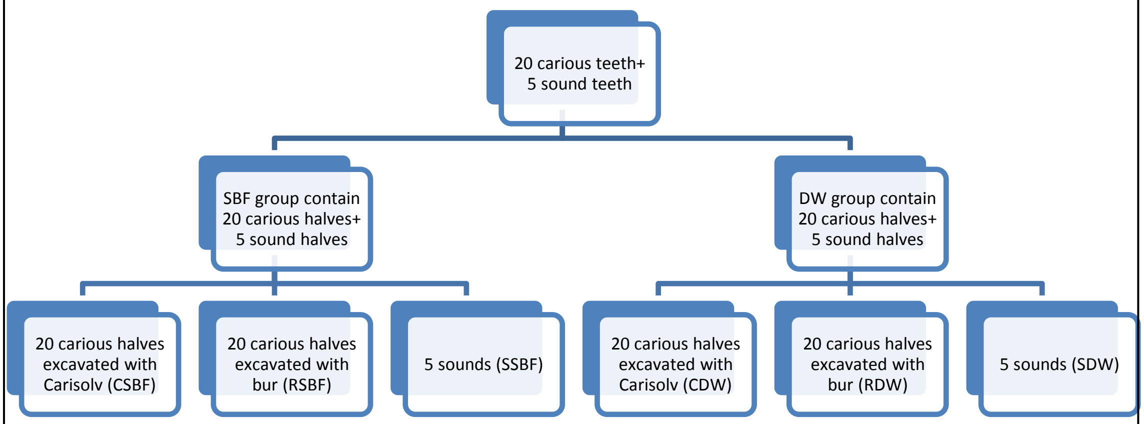


Figure 4-4: Groups and subgroups of the experiment.

4.4.2 Chemical and Mechanical Analysis:

4.4.2.1 Raman Spectroscopy

The samples were investigated using a Renishaw inVia Raman microscope (Renishaw Plc, Wotton-under-Edge, UK) (Figure 4-5), running in Streamline^{TM} scanning mode used to scan the sectioned surfaces with a 785-nm diode laser (100% laser power) focused using a 20/0.45 air objective. The signal was acquired using a 600 lines/mm diffraction grating centred at 900 cm^{-1} and a CCD exposure time of two seconds. The microscope was calibrated using an internal silicon sample with a characteristic band at 520 cm^{-1}. Five Raman maps of the MTA / caries-affected dentine interface were recorded for each sample at 24-hour and 14-day intervals. On day 14, an extra polishing step was performed before Raman scanning for 30 seconds with 4000 grit carborundum paper to remove any surface precipitates.

Figure 4-5: Renishaw inVia Raman microscope (Renishaw Plc, Wotton-under-Edge, UK)

The Raman map started in the MTA and extended to the dentine to cover the surface area of 297×256.5 μm^2 of the interface (Figure 4-6), and contained 10,450 spectra acquired with 2.7 µm resolution. For the analysis of Raman peaks, the maps were transferred to an in-house program designed to fit the spectra and then generate grey-scale images of phosphate peak intensity (mineral peak intensity (MPI)) at 959 cm^{-1} (PO_4^{-3} $\nu1$) (Figure 4-7), across the sound and demineralised dentine areas. The average peak intensity of PO_4^{-3} in the first 50µm in dentine from the MTA / dentine interface was calculated as shown in Figure 4-8.

Figure 4-6: The Raman map started in the MTA and extended to the dentine to cover the surface area of 297×256.5 µm^2 of the interface

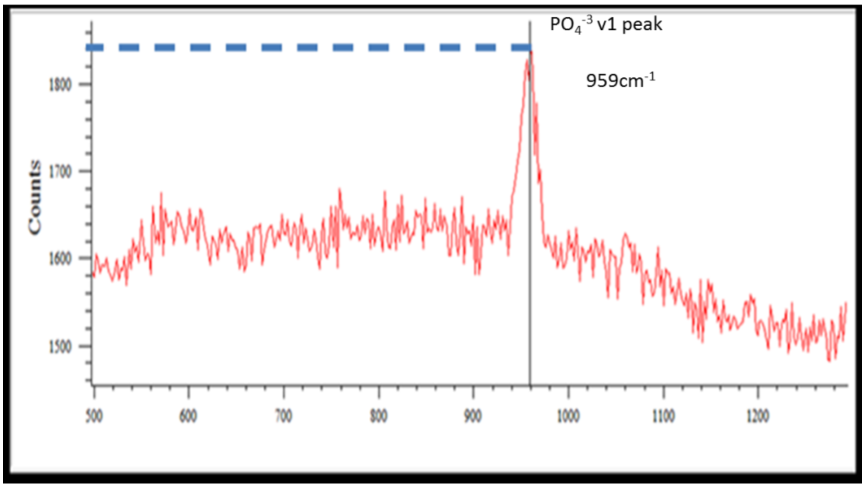


Figure 4-7: PO_4^{-3} *v1* peak analysis at 959cm^{-1} using Renishaw inVia Raman microscope to scan dentine/MTA interface at 24 hours and 14 days.

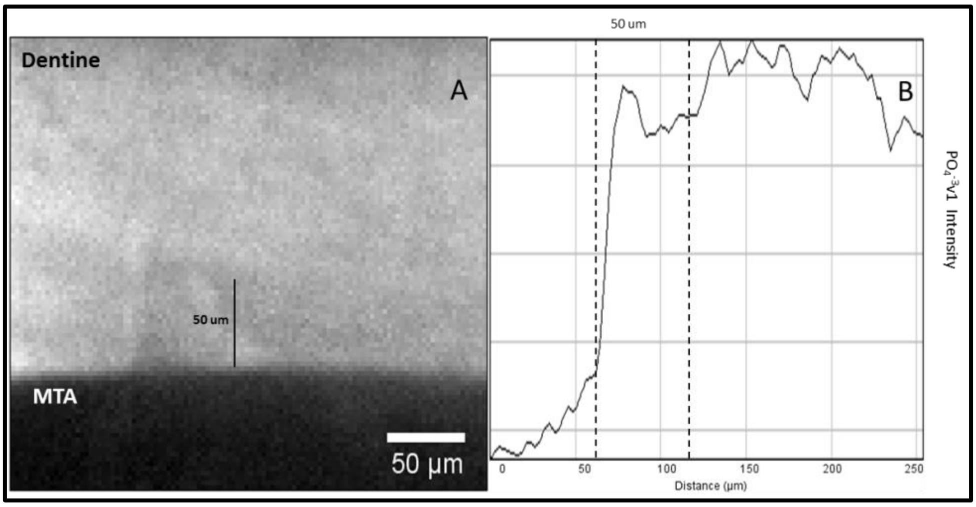


Figure 4-8: (A) Representative grey-scale image of Raman phosphate peak intensity at 959 cm^{-1} including MTA and dentine areas of the scanned map with 50 µm measured area from the interface. (B) Depth profile of phosphate peak intensity along the scanned area illustrating phosphate peak intensity in the interfacial 50 µm.

4.4.2.2 Knoop Microhardness

A Struers Duramin micro-hardness tester (Struers Ltd., Denmark) with a Knoop diamond indenter was used with a load of 25g applied for 10s. The indentations were imaged with a 40/0.65 NA objective and the Knoop values were calculated using the manufacturer's software supplied. Two sets (n=10/set) of measurements were recorded; the first set was recorded at 50 µm from the MTA / dentine interface in dentine with 150 µm intervals. The second set of measurements was recorded at 150 µm from the MTA / dentine interface in dentine also recorded with 150µm intervals. Measurements were then averaged to calculate the micro-hardness of each sample at 50 and 150 µm from the interface, and measurements were repeated on day 14, as shown in **Figure 4-9**.

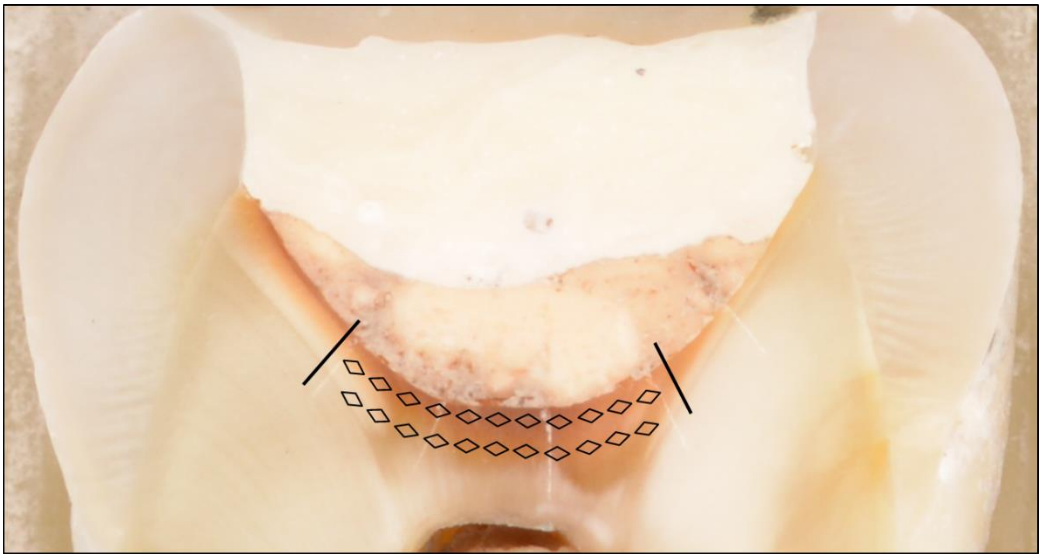


Figure 4-9: Microhardness test at two levels 50 and 150 microns from the interface between MTA and dentine.

4.4.3 Scanning Electron Microscopy (SEM)

One tooth with a carious lesion extending up to half of the dentine depth was sectioned mesiodistally and each half was excavated with either chemo-mechanical caries removal technique (Carisolv™ gel) or rotary burs. A scanning electron microscope (FEI Co. Ltd., Cambridge, UK) was used to examine the ultrastructure of the excavated surfaces in each tooth (accelerating voltage of 3.5 and 10 kV, working distance of 10 mm, magnifications: 2656 and 3000 under high vacuum conditions).

4.4.4 Statistical Analysis

Statistical analysis was conducted using the SPSS statistical package (version 20; SPSS Inc., IBM, Chicago, IL, USA). Data were tested for normality using Q–Q plots and Shapiro–Wilk tests. Paired sample T-test was used to compare between day one and day 14 in each subgroup to assess the time factor. Independent sample T-test was used to compare between techniques (Carisolv and rotary subgroups) to assess technique factor and depth factor in microhardness. One-way analysis of variance (ANOVA) was used to compare between Carisolv^{TM}, rotary and sound subgroups to assess the technique factor. Significant level was assumed at p=0.05.

4.5 Results

4.5.1 Mineral Content

The four internal vibration modes of phosphate ion (PO_4^{-3}) within dentine were observed as peaks at 433 cm^{-1} (symmetric bending vibrational mode – PO_4^{-3} *v2*), 579 cm^{-1} (asymmetric bending vibrational mode – PO_4^{-3} *v4*), 959 cm^{-1} (symmetric stretching vibrational mode – PO_4^{-3} v1) and 1043 cm^{-1} (asymmetric stretching vibrational mode – PO_4^{-3} *v3*). All peaks were observed within sound and demineralised dentine's spectra with no difference in their positions. The strongest peak along sound and demineralised dentine spectra were that of PO_4^{-3} *v1* at 959 cm^{-1}.

4.5.1.1 DW Group

Time factor: Paired sample t-test showed that the average phosphate peak intensity (MPI) within 50 µm from the interface decreased in each subgroup, in CDW (from 30.93±2.92 to 26.83± 4.47) (mean±ste) (p=0.19) and RDW (from 56.21±2.85 to 39.22± 3.51) (mean±ste) (p=0.001) and SDW (from 146.3±13.4 to 109.4±18.2) (mean±ste) (p=0.03) after 14 days.

Technique factor: At baseline, there was a significant difference between CDW and RDW at day one using independent sample t-test (p<0.001), implying that less mineral phosphate content was present within the Carisolv^{TM} gel excavated dentine initially. After 14 days, also there was a significant difference between CDW and RDW (p=0.03). ANOVA showed there was a significant difference between CDW, RDW and

SDW at day one and 14 days (p<0.001) and (p<0.001) respectively. Figure 4-10 shows MPI of different subgroups in DW.

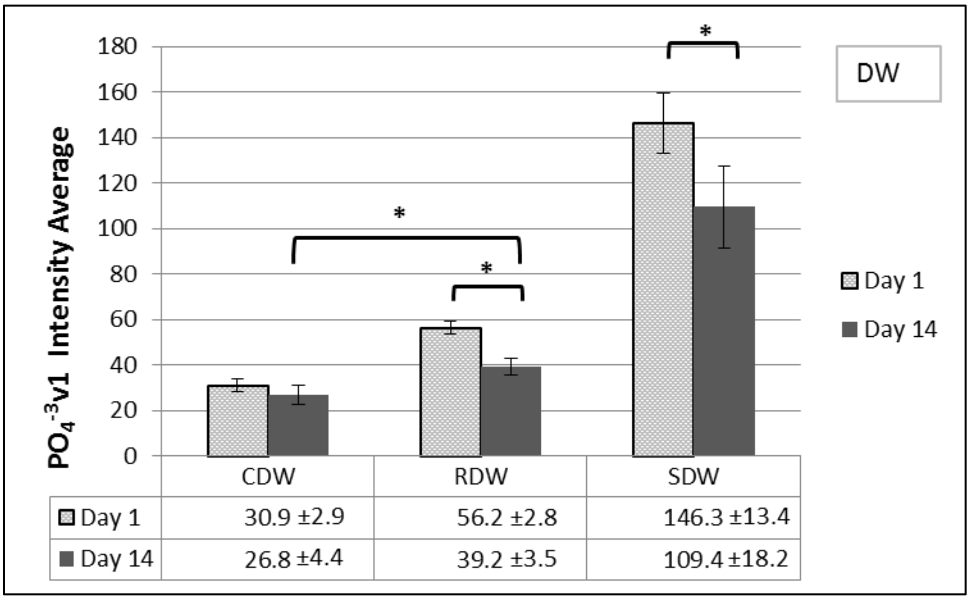


Figure 4-10: Bar graph representing mean±SE of phosphate peak intensity (MPI) average within 50 µm from the interface in teeth stored in DW and excavated either with Carisolv™ or rotary burs, (*) indicate a statistically significant difference.

4.5.1.2 SBF Group:

Time factor: Paired sample t-test showed that there was a significant increase in MPI in each subgroup after 14 days, except in SSBF, in CSBF (from 45.27± 3.20 to 109.79± 16.61 (mean±ste) (p=0.001)), RSBF (from 74.23±10.40 to 132.64±21.03 (mean±ste) (p=0.01)) and SSBF (from 142.61±8.94 to 152.46±7.14 (mean±ste) (P=0.53)).

Technique factor: At baseline, there was a significant difference between CSBF and RSBF at day one using independent sample t-test (p=0.01). After 14 days, although there were 142.5% and 78.6% increases in MPI in CSBF and RSBF subgroups, respectively, there was no significant difference between the CSBF and RSBF subgroups after 14 days using independent sample t-test (p=0.40). ANOVA showed that there was a significant difference between CSBF, RSBF and SSBF at one day ($p<0.001$). However, there was no significant difference in MPI between CSBF, RSBF and SSBF at 14 days interval ($p=0.44$). Figure 4-11 shows MPI of different subgroups in SBF.

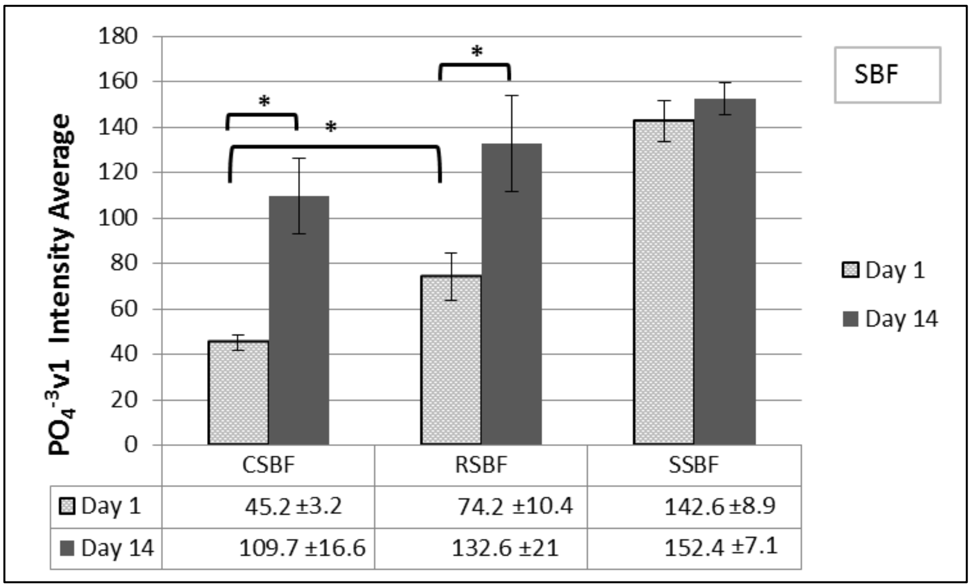


Figure 4-11: Bar graph represents mean±ste of phosphate peak intensity (MPI) average within 50 µm from the interface in teeth stored in SBF and excavated either with Carisolv™ or rotary burs; (*) indicates statistically significant difference.

4.5.2 Knoop Microhardness

4.5.2.1 DW Group

Depth level: Independent sample t-tests showed there were no significant differences in Knoop hardness number (KHN) between 50 and 150 µm points in CDW at both day one ($p=0.1$) and day 14 ($p=0.1$). They also showed there were no significant differences in KHN between 50 and 150 µm points in RDW at both day one and day 14 ($p=0.09$ and $p=0.09$, respectively).

Time factor: Paired sample t-test showed no significant difference in KHN in CDW and RDW in each point at 50 and 150 µm after 14 days (p values for CDW for 50 and 150 µm were p=0.9 and p=0.5, respectively; RDW p values for 50 and 150 µm were p=0.6 and p=0.7 respectively). SDW showed a significant decrease in KHN after 14 days (p<0.001).

Technique factor: Independent sample t-test showed significant differences between CDW and RDW at 50 and 150 µm points (p<0.001 and p<0.001 respectively) at day one. Also at day14, there were significant differences between CDW and RDW at 50 and 150 µm points (p<0.001 and p<0.001, respectively). ANOVA showed significant differences between CDW, RDW and SDW at day one and day 14 (p<0.001 and p<0.001, respectively). Figure 4-12 shows KHN of all subgroups in DW.

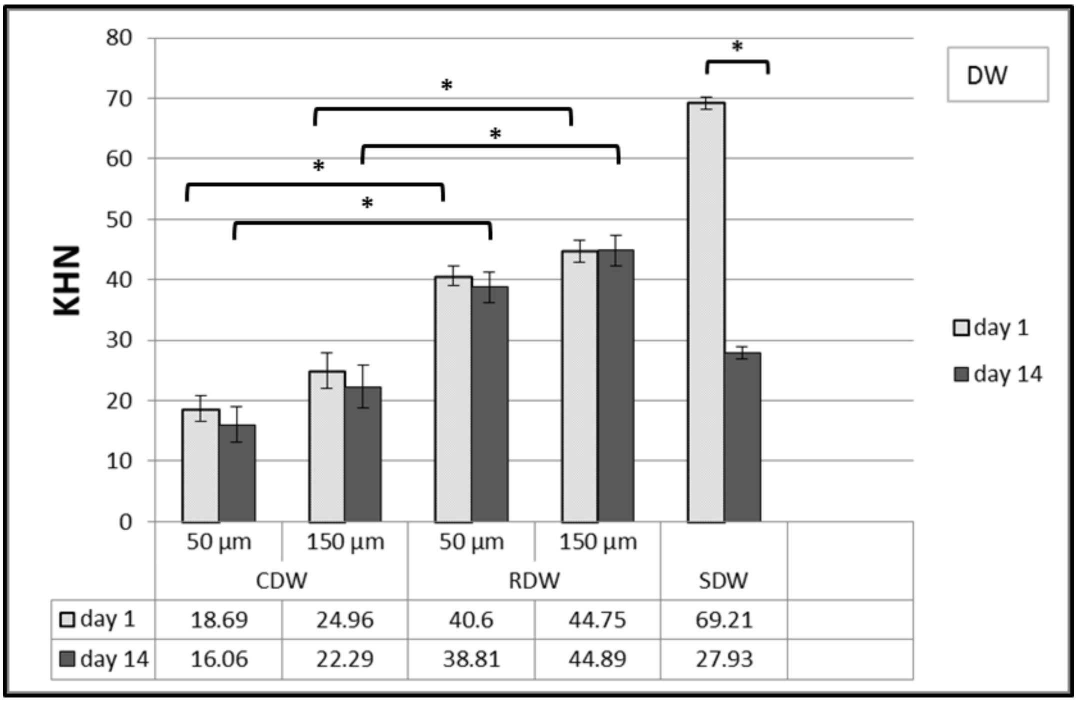


Figure 4-12: Bar graph showing mean±ste of KHN at 50 and 150 um from the interface between MTA and dentine in teeth stored in DW; (*) indicates statistically significant difference.

4.5.2.2 SBF Group:

Depth level: Independent sample t-tests showed there was a significant difference in KHN measurements between 50 and 150 µm in CSBF at day one ($p=0.002$); however, there was no significant difference at day 14 ($p=0.17$). Also, there was no significant difference in KHN between 50 and 150 µm in RSBF at day one and day 14 ($p=0.23$ and $p=0.30$, respectively).

Time factor: Paired sample t-test showed a significant increase in KHN between day one and day 14 in CSBF and RSBF at 50 and 150 µm (p-values for CSBF for 50 and 150 µm were $p<0.001$ and $p=0.04$, respectively; RSBF p-values for 50 and 150 µm were $p<0.001$ and $p=0.001$, respectively). SSBF showed no significant change in KHN between day one and day 14 ($p=0.67$).

Technique factor: Independent sample t-test showed significant differences between CSBF and RSBF at 50 and 150 µm points ($p<0.001$ and $p=0.005$, respectively) at day one. Also at day 14, there were significant differences between CSBF and RSBF at 50 and 150 µm points ($p<0.001$ and $p<0.001$, respectively). ANOVA showed significant differences between 50 µm measurements of CSBF, RSBF and SSBF at day one and day 14 ($p<0.001$ and $p<0.001$ respectively). Figure 4-13 shows KHN of all subgroups in SBF.

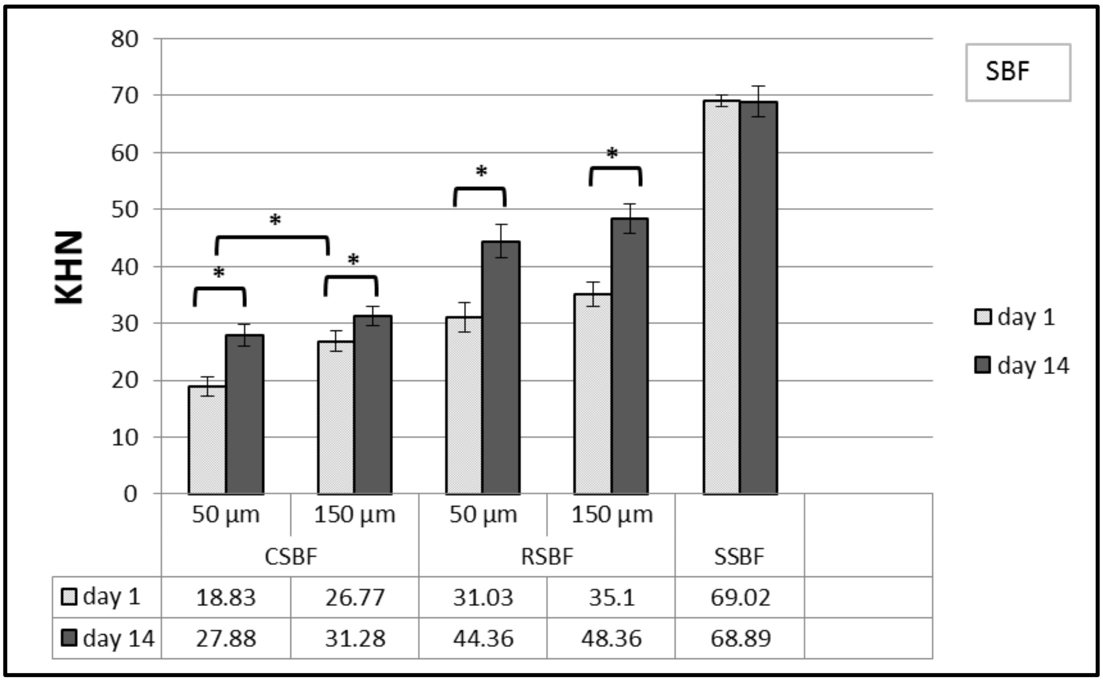


Figure 4-13: Bar graph representing mean± ste of KHN at 50 and 150 µm from the interface between MTA and dentine in teeth stored in SBF; (*) indicates statistically significant differences.

4.5.3 SEM

SEM images of carious dentine surface excavated with Carisolv^{TM} gel showed the openings of dentine tubules that were partially occluded with smear layer. SEM images of dentine surface excavated with rotary burs show complete occlusion of dentine tubules opening with smear layer as shown in Figure 4-14.

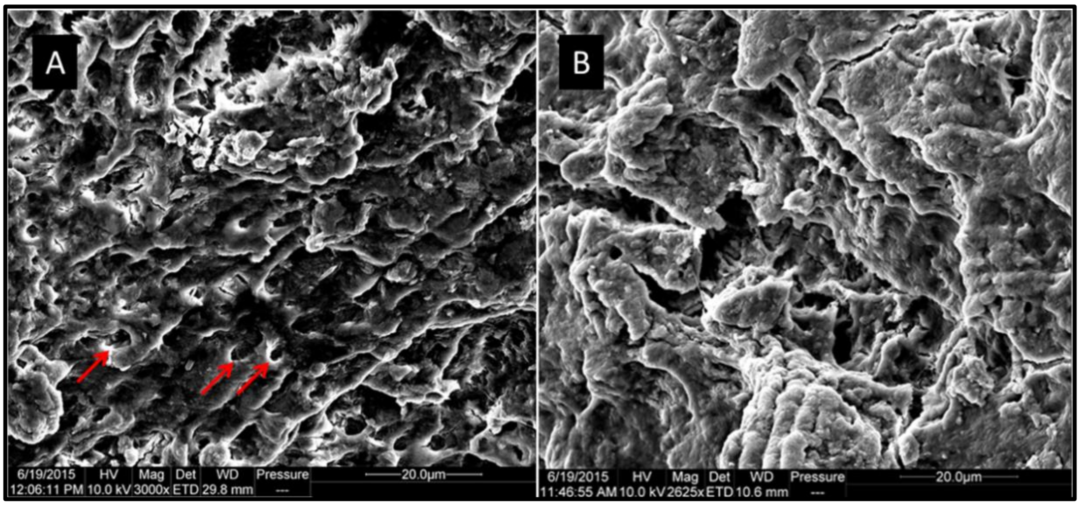


Figure 4-14: SEM images of excavated dentine surface. A) Carisolv^{TM} excavated dentine surfaces at a magnification of 3000x; arrows point to open dentine tubule orifices. B) Rotary burs excavated dentine surface, show accumulation of smear layer occluding opening of dentine tubules.

4.6 Discussion

Raman and Knoop microhardness data at baseline showed significant differences in the mineral content and hardness of dentine after excavation with Carisolv^{TM} gel compared to the excavation with rotary burs in the specimens of both DW and SBF groups. Carisolv^{TM} leaves CAD with lower MPI and KHN than that excavated with the rotary burs. This confirms that Carisolv^{TM} gel retains more CAD compared to rotary burs in the cavity floor, which is in agreement with other studies that described the selective removal of Carisolv^{TM} (Banerjee *et al.* 2000b, Splieth *et al.* 2001). Rotary bur excavation led to a cavity end point located within more highly mineralised dentine because of non-selective cutting which reflected the higher mineral and microhardness values observed at baseline measurements with Raman spectroscopy and Knoop microhardness test; this concurs with findings of other studies (Magalhães *et al.* 2006, Mollica *et al.* 2012, Hamama *et al.* 2013). Varied selectivity between two excavation techniques produces cavities with different depths in the CAD layer which have a gradual increase in hardness toward sound inner dentine and pulp wall (Ogawa *et al.* 1983).

After two weeks' storage in DW, mineral content in both RDW and SDW decreased significantly because of the pH of DW (pH=6.5) which could lead to a potential dissolution of calcium and phosphate ions from tooth structure into the solution (Habelitz *et al.* 2002). The lack of phosphate ions, a major constituent for mineral deposition, might lead to a reduction in microhardness and mineral content of dental hard tissues (Habelitz *et al.* 2002). The transport of ions is considered a major factor and essential in the demineralisation / remineralisation process (Kawasaki *et al.* 2000). In Carisolv™ gel-treated dentine, the overall content of mineral was low initially, which may impede the process of ion exchange with DW. This results in a non-significant decrease in mineral content compared to what happens in the rotary and control subgroups, which both have a higher initial mineral content which may enable robust ion exchange.

Storage in SBF provided an abundant source of phosphate ions in addition to the calcium ion sources from the teeth and MTA, which resulted in a mineral deposition in both CSBF and RSBF, and it was not significantly different from the level of mineral found in SSBF after 14 days. It has been suggested that calcium ions from MTA interact with phosphate ions from SBF, producing hydroxyapatite crystals in the MTA-dentine interface (Sarkar *et al.* 2005). Others have suggested that calcium-deficient carbonated apatite crystallites formed from the transformation of amorphous calcium phosphate phase is a key intermediate in biological apatite formation (Tay *et al.* 2007, Reyes-Carmona *et al.* 2009). The probability of mineral precipitation in a mineral-depleted CAD after selective / non-selective caries excavation is apparent from the results of this study. Control samples with higher mineral content did not acquire any further mineral deposition after 14 days, in contrast to the intermediate mineral deposition rate in samples excavated with rotary burs and high mineral deposition rate in samples excavated with Carisolv^{TM} gel. It has been found that there is a significant effect of initial mineral level in the surface of the lesion on the subsequent mineral deposition rate and distribution (Kawasaki *et al.* 2000). The first null hypothesis has been accepted as the mineral content in the retained dentine excavated with Carisolv^{TM} gel did not significantly differ from that of retained dentine excavated with rotary burs or that of the sound dentine after two weeks.

There was a reduction in the hardness of sound dentine stored in DW compared to that stored in SBF after 14 days. This could be due to the absence of both phosphates and calcium ions in SBF and MTA, respectively, which may lead to the dissolution of ions from tooth structure to achieve ionic equilibrium in solution. It is known that apatite precipitation requires the initial step of ionic dissolution and the growth and nucleation of the apatite precipitates are proportional to the concentration of the available ions (Weng *et al.* 1997, Khor *et al.* 2003). Although the hardness of dentine excavated with the rotary burs was significantly higher than that excavated with Carisolv^{TM} gel at baseline and after the storage period, dentine substrate after both excavation methods did not attain the hardness of sound dentine after 14 days' storage in SBF.

Although mineral content of CAD after excavation with both techniques was comparable to that of sound dentine after two weeks, CAD did not improve its hardness compared to that of sound dentine. Fusayama suggests that passive mineral precipitation within dentine tubules might not fully restore the mechanical properties of dentine, possibly due to whitlockite (β-octocalcium phosphate, OCP) rather than apatite being precipitated (Fusayama 1991). However, in the present study, the deposition of hydroxyapatite in dentine is specific as the characteristic Raman peaks of other possible minerals such as OCP, dicalcium phosphate dihydrate (DCPD), tricalcium phosphate (TCP) or amorphous calcium phosphate (ACP) show the PO_4^{-3} *v1* band at different wave number shifts (cm^{-1}) (Koutsopoulos 2002).

Other studies showed similar results, as there was a non-linear correlation between mineral density and mechanical properties of wet remineralised dentine, especially in the absence of intra-tubular remineralisation (Kinney *et al.* 2003). In addition, it is known that hardness of CAD is lower than sound dentine, because of a decrease in number and size of apatite crystals in intertubular dentine in CAD due to demineralisation (Fusayama 1993). The second null hypothesis was rejected because microhardness of dentine excavated with both techniques was incomparable and lower than that of sound dentine after storage in SBF.

In the present study, the storage solution had a significant impact on the physiochemical characteristics of stored dentine and this is in agreement with other studies (Habelitz *et al.* 2002, Sauro *et al.* 2011, Schwendicke *et al.* 2015). The factors that influence the bioavailability of calcium and phosphate ions play a substantial role in promoting ion precipitation in the demineralised CAD. The saturated level of these ions in SBF promotes the precipitation of ion clusters in the voids of the demineralised tissue.

Naturally, saliva provides bioavailable calcium and phosphate ions to remineralise demineralised enamel (Cochrane *et al.* 2010). Although fluoride plays an important role in enamel remineralisation by controlling calcium and phosphate ion uptake, dentine remineralisation is difficult to achieve in the presence of fluoride (Damen *et al.* 1998). Surface examination revealed remineralisation on the enamel surface but not on the dentine surface with identical remineralisation parameters (Fan *et al.* 2009). Extracellular matrix phosphoproteins such as DPP (dentine phosphoprotein) can regulate growth and inhibition of apatite nucleation in collagenous tissue. It binds to collagen surfaces and lowers the interfacial energy for hydroxyapatite nucleation. However, others inhibit crystal growth by producing an electrostatic repulsion of inorganic phosphate ions (George and Veis 2008).

In dentine remineralisation, the availability of these ions can be compromised by the presence of translucent dentine at the advancing front of the lesion, which will minimise saturated fluids levels to enhance the remineralisation process of dentine. In addition, the presence of individual plasma proteins fractions and several different types of bacteria can influence the permeability of fluids across the dentine (Pashley *et al.* 1982).

The dissimilar SEM patterns of the dentine surfaces obtained in the two techniques might have an effect on the mineral precipitation, hardness and dentine permeability *in vivo*. It has been found that microleakage decreased in teeth restored with resin composite after treatment with Carisolv™ gel, due to the smear layer-free irregular surface that can enhance adhesion to adhesive restorative materials as confirmed by SEM (Yamada *et al.* 2006).

The rationale for the use of MTA in this study (pH 11.7) was to have a bioactive material that can promote calcium phosphate apatite precipitation in CAD in a phosphate-containing fluid (Tay *et al.* 2007, Reyes-Carmona *et al.* 2009). The documented sealing ability and biocompatibility of MTA could make it a suitable choice to prevent an inflammatory response in dental pulp after excavation of dental caries in deep carious lesions (Prati and Gandolfi 2015). In addition to the role of MTA, dentine bioactive components (non-collagenous proteins, glycosaminoglycans and TGF-β1) have been proven *in vitro* and clinically to have the potential to regulate dentine repair and regeneration which include remineralisation (Tomson *et al.* 2007, Ferracane *et al.* 2010). The presence of MTA as a source of calcium and hydroxyl ions and as an initiator of dentine bioactive component release could have a reservoir effect on the samples stored in DW. This could create a chemical equilibrium, raise pH above 7 (Duarte *et al.* 2003), prevent further dissolution of ions from tooth structure into DW and result in a non-significant decrease in hardness of samples excavated with Carisolv™ or rotary bur compared to the significant drop in control subgroup, which has no MTA. It has been reported that calcium silicate cement can produce a "mineral infiltration zone" in underlying dentine because of the influx of ions from the cement to the dentine that happens after partial degradation of collagen in the interfacial dentine; this has been detected *in vitro* by a two-photon autofluorescence microscope with second harmonic generation and confocal laser scanning microscope (Atmeh *et al.* 2012). Also, there was a clinical finding of a radiopaque layer in dentine under calcium silicate cement restorations in patients after 12 months of follow-up, which may imply mineral exchange (Hashem *et al.* 2015).

There is variability between naturally demineralised dentine lesions compared to artificially demineralised dentine used in many previous published works. These natural lesions could be more difficult to remineralise due to their variation in depth, stage and degree of tubular occlusion (Pugach *et al.* 2009). Although many studies have been conducted on remineralisation of artificially demineralised dentine (Petersson and Kambara 2004, Tay and Pashley 2009, Qi *et al.* 2012), remineralisation of natural carious dentine represents the most realistic model of the clinical condition per se.

The split tooth model may not simulate the natural situation because of the direct contact between the remineralising solution and the sectioned surface of the tooth. Alternatively a whole tooth model combined with the circulation of simulated body fluid (SBF) in the pulp chamber using peristatic pump (Chen *et al.* 2015), would be the ideal model to simulate the real clinical environment of deep caries during vital pulp therapy as far as possible. However, there are some limitations in the whole tooth model represented by the impossibility of recording repeatable measurements of the microhardness of the demineralised/remineralised dentine, also, the inability to implement the two excavation methods in the whole tooth model cavity without a possibility of interfering between their excavation limits on the floor of the cavity. Many studies that assessed dentine remineralisation used split tooth model/dentine discs accompanied with direct contact to the remineralisaing solution.

4.6.1 Conclusions

Within the limits of the present study, it was found that remineralisation of natural CAD is possible after excavation either with Carisolv^{TM} gel or rotary burs in the presence of MTA. Results are confirming the selective, minimally invasive nature of Carisolv^{TM} gel in comparison to the rotary bur. Also, there is a significant influence of the type of storage solution on the remineralisation of dentine *in vitro*. Moreover, MTA can maintain dentine hardness in slightly acidic aqueous solution.

4.6.2 Clinical Significance

The use of Carisolv^{TM} gel provides an alternative to rotary burs in terms of preserving tooth structure and ability to remineralise valuable tooth structure, a clinical advantage in minimally invasive dentistry.

Chapter 5 Assessment of a Minimally Invasive Caries Excavation Protocol in Teeth with Reversible Pulpitis: a Randomised Controlled Clinical Trial.

5.1 Introduction

There are many alternatives available for treatment of deep carious lesions, ranging from vital pulp therapies to root canal treatment. Although many dental practitioners favour complete removal of caries and jeopardise exposure of the pulp rather than leaving a layer of caries and utilise indirect pulp capping (IPC) to restore the tooth (Oen *et al.* 2006, Hilton 2009), teeth with pulp exposures are usually associated with severe post-operative symptoms and bacterial microleakage with unpredictable results compared to non-exposed cavities (Barthel *et al.* 2000, Murray *et al.* 2002b, Dammaschke *et al.* 2010, Bjørndal *et al.* 2010).

Selective caries removal in IPC can be considered a mode of vital pulp therapy which has the potential to not jeopardise the pulp sensibility and function of teeth with deep carious lesions by incompletely excavating the carious dentine biomass (Tziafas *et al.* 2000). Studies reported favourable outcomes in terms of preserving tooth vitality by selective removal of carious biomass in these teeth (Maltz *et al.* 2007, Gruythuysen *et al.* 2010). Also, incomplete carious dentine removal significantly improves pulp preservation after a three-year period compared to stepwise excavation, with survival rates of 91% and 69% in incomplete and stepwise excavations respectively (Maltz *et al.* 2012). However, it is important that IPC procedures are not performed in teeth with signs and symptoms of irreversible pulpitis (Al-Zayer *et al.* 2003, Gruythuysen *et al.* 2010, Hashem *et al.* 2015). Since there are drawbacks associated with the diagnostic clinical and radiographic methods (Sigurdsson 2003), it is challenging to determine the pathological and inflammatory status of the pulp accurately in these cases to accomplish the treatment of choice with foreseeable outcomes. Pulpal status diagnosis is subject to interpretation of subjective patient's signs and symptoms, and results of sensibility test and radiographic examination (Bjørndal 2002).

Deep dentine caries excavation procedures should be performed conventionally under regular aseptic conditions with or without the use of magnifying loupes and rubber dam (Oliveira *et al.* 2006, Maltz *et al.* 2007, Maltz *et al.* 2012). Since the early 1990s, operating microscopes have been introduced in the field of endodontics. Studies and reviews reported the advantage of utilising high-power magnification in increasing the success and improving management in surgical and non-surgical endodontics compared to the traditional approach without magnification (Setzer *et al.* 2012, Kim and Baek , Schwarze *et al.* 2002, Baldassari-Cruz *et al.* 2002, Ömer Görduysus *et al.* 2001). The ability of a clinician to remove caries from the lesion depth may be hindered by the higher risk of iatrogenic pulp exposure, especially in those lesions that penetrate more than three-quarters of dentine thickness. Therefore using an operating microscope has the potential to improve visibility, accessibility and preservation of tooth structure during cavity preparation in deep carious lesions.

In conventional caries removal procedures, mechanical rotary burs and/or hand excavators are used to remove the carious biomass (Bjørndal *et al.* 1997, Bjørndal 2011, Orhan *et al.* 2010), however there are many disadvantages associated with the use of rotary burs, such as heat generation, mechanical irritation, non-selective removal of caries and excessive removal of tooth structure (Banerjee *et al.* 2000b). Minimally invasive caries removal techniques such as Carisolv^{TM} gel (Rubicon Lifesciences, Gothenburg, Sweden) could provide effective caries removal and prevent excessive tooth structure loss during cavity preparation (Banerjee *et al.* 2000b), in addition to its inherent anti-bacterial effects on carious dentine (Lager *et al.* 2003). Therefore, using Carisolv^{TM} gel could reduce the possibility of failures in the treatment of deep carious lesions. The use of the operating microscope, combined with Carisolv^{TM} gel in pulp protection procedures, has the potential to further reduce sound tooth structure removal and has, to date, not been compared with conventional caries removal protocols in a clinical trial in deep dentine carious lesions.

In this study, the indirect pulp capping bioactive material used was MTA, which is a tri-calcium silicate-based cement. MTA has been recognised as a bioactive (apatite-forming) material when it is exposed to saline (Enkel *et al.* 2008, Gandolfi *et al.* 2014), as well as being hard tissue conductive, and biocompatible (Moretton *et al.* 2000, Ribeiro *et al.* 2006). Moreover, it has low solubility (Fridland and Rosado 2005) and possesses some antibacterial activity (Zhang *et al.* 2009).

Recent laboratory and clinical investigations have revealed that cone-beam computed tomography (CBCT) can detect the presence of peri-radicular lesions better than conventional periapical radiographs (S. Patel *et al.* 2012). In teeth with irreversible pulpitis, the detection of the prevalence of periapical lesions was higher with CBCT than periapical radiographs (Abella *et al.* 2012). Also, it has been reported that indirect pulp capping of teeth presented with clinical reversible pulpitis signs and symptoms but presenting with an initial CBCT periapical lesion had a failure rate of 63%, whereas teeth with no initial lesion had a failure rate of 16% (Hashem *et al.* 2015). In the present study, teeth presenting with reversible pulpitis were selected for IPC only if presenting with no CBCT evidence of periapical radiolucency.

5.1.1 Aims and objectives

This randomised controlled clinical trial following CONSORT guidelines (Appendix 2) investigated the tooth-pulp response to selective deep caries removal carried out using two different clinical protocols in teeth presenting with signs and symptoms of reversible pulpitis. In group A, a “control” protocol was applied which involved the use of the mechanical rotary burs without magnification (naked eye) for excavation of deep carious dentine, and in group B, a “minimally invasive” protocol was used which involved the use of a dental operating microscope and Carisolv™ system in excavation of deep carious dentine in teeth with reversible pulpitis. The association between the one-year outcomes and different clinical attributes such as symptoms intensity, cavity size, age, gender and tooth type were also assessed.

In addition, this trial investigated the spectrum of detectable bacterial species and loads associated with the superficial and deep layers of the carious dentine tissue using a non-culture-based technique (16S rRNA gene qPCR and next-generation sequencing) to analyse carious dentine samples collected during the operative procedure. Aims, materials and methods, results and discussion of the microbiological analysis are described in detail in Chapter 6.

The objective of this trial was to assess the effectiveness of each protocol in preserving pulp vitality and PA tissue health in the treated teeth after a 12-month follow-up interval, using standardised clinical and radiographic examination and CBCT assessment to overcome the radiographic evaluation shortcomings associated with 2-dimensional PA radiographs.

5.1.2 Null hypothesis

The null hypothesis investigated was there is no difference clinically and radiographically between the conventional "control" protocol and the minimally invasive "experimental" protocol effectiveness in both preserving pulp sensibility and maintaining PA radiographic health after a 12-month follow-up interval in teeth with signs and symptoms of reversible pulpitis.

5.2 Materials and Method

5.2.1 Study design and ethical approval

The study was designed to be a single-blind, two-arm, randomised controlled clinical trial (RCT), with clinical and radiographic examiners blinded to which protocol was used. This RCT compared two protocols for treatment of teeth with deep caries that involve 2/3 or more of dentine thickness radiographically. Mineral trioxide aggregate MTA®caps (Acteon, Pierre Rolland, Merignac, France) was used as an indirect pulp capping (IPC) agent for teeth in both groups. The study was reviewed and approved by the London-South East research ethics committee (14/LO/0880) and the GSTFT NHS R&D office (Appendix 3 and 4). The study was conducted in compliance with the principles of the Declaration of Helsinki and good clinical practice. Patient information sheets containing a detailed explanation of the purpose of the study, treatment protocol, follow-up examinations and total radiation dose anticipated during the study (Appendix 5) were distributed and informed written consent (Appendix 6) was obtained prior to the implementation of the study. The trial registered in ClinicalTrials.gov registry (NCT03071588).

5.2.2 Sample size

This study was designed to have 80% power (type 2 error of 20%) and a probability of Type I error $\alpha = 0.05$ to detect a difference between the two protocols whose percentage of failed outcomes were expected to be 1% versus 20% after a one-year follow-up. Using a two-tailed z test comparing two independent proportions, a conventional design of the study required a sample size of 40 restorations per technique and, to compensate for an expected loss to follow-up of 10%, the power calculations assigned 44 to each technique (88 restorations in total).

5.2.3 Recruitment and randomisation

The patients were recruited from the acute dental care and endodontics consultation clinics at King's College London Dental Institute at Guy's Hospital, London, UK. Randomisation was performed by the Biostatistics Unit, King's College London Dental Institute. A stratified randomisation of teeth was used in between groups. The cavity size (one, two or more than two wall cavity involvement) was considered as a prognostic factor to be balanced during allocation of patients into each study group. Eventually, each group included identical percentages of cavity wall involvement categories (10% one wall, 80% two walls and 10% more than two walls).

This study recruited patients presenting with signs and symptoms of reversible pulpitis in teeth with deep carious lesions. The medical and dental history was taken and the signs and symptoms of the patient registered; the patient's symptoms were categorised into mild and severe. Mild symptoms were defined as a mild short duration (no more than 10-15 seconds) sharp pain caused by cold, sweet and hot stimuli that subsided after removal of the stimulus. Severe symptoms were defined as severe pain that is not lingering after removal of stimulus for more than one minute and not spontaneous

(Levin *et al.* 2009). Teeth with a throbbing, postural or spontaneous history of pain were excluded as these symptoms were considered indicative of irreversible pulpitis. Teeth with a previous history of pain were also excluded. Table 5-1 describes inclusion and exclusion criteria.

Table 5-1: Inclusion and exclusion criteria of patients

Inclusion criteria	**Exclusion criteria**
1) Patients either male or female over the age of 16 (who can consent for themselves) in good general health. 2) A minimum of one deep carious lesion (occlusal or proximal) penetrating 2/3 or more of dentine thickness in the periapical radiograph. 3) A positive response to electric pulp test or cold test that lasts no more than one minute. 4) Teeth with symptoms of reversible pulpitis (mild to severe response to cold/sweet or hot stimuli that resolves within seconds after removal of stimuli (Levin *et al.* 2009). 5) Posterior teeth only	1) Patients with clinical symptoms of irreversible pulpitis. 2) The presence of fistulas or swelling. 3) Anterior teeth. 4) The presence of radiolucency or thickening of the periodontal spaces in preoperative periapical radiographs. 5) External or internal root resorption. 6) Mobile teeth or teeth tender to percussion. 7) Pregnant women, in view of requirements for radiographs. 8) Patients younger than 16. 9) Patients unable to give consent. 10) Patients who have been administered antibiotics in the previous month.

5.2.4 Clinical intervention

Operative procedures were undertaken by thirty members of staff and endodontic residents. The operators have and experience background of 5 years in dental practice on average. The operators were trained on carious extracted teeth using both protocols before commencing the trial to ensure standardisation of both operative protocols. The assessment methods included pulp sensibility tests (thermal and electrical), palpation of surrounding soft and hard tissue and percussion test, along with looking for the presence of signs of inflammation (pain, abscess, sinus tract, and abnormal mobility). Pulp sensibility tests included thermal test (Roeko Endo-Frost, Coltène/Whaledent, Germany) and electric pulp test (Kerr Vitality Scanner 2006; SybronEndo, Orange, CA, USA) using the value of the healthy contralateral tooth as a standard. Periapical (PA) radiographs and cone-beam computed tomography (CBCT) were used to assess the tooth periapical area health/pathosis at baseline (T0) by an experienced endodontist familiar with the use of CBCT imaging to exclude all cases with the width of radiolucency exceeding two times the periodontal ligament (PDL) space (Bornstein *et al.* 2011). Caries removal and microbiological study samples collections were carried out under local anaesthetic and rubber dam isolation.

5.2.4.1 Carious tissue removal

In Group A (*"conventional caries removal"* or "control"), appropriate access through the cavitated enamel was gained using a high-speed TA-98 handpiece (W&H Dentalwerk GmbH, Bürmoos, Austria) with carbide and diamond burs and copious water jet cooling. Superficial carious dentine tissue samples were collected with a sterile spoon excavator (Ash G5; Claudius Ash Ltd., Potters Bar, UK) from superficial carious dentine and transferred into a pre-weighted sterile Eppendorf tube to be used for the microbiological analysis. Then the caries-infected dentine was removed using carbon-steel rose-head burs (Ash Instruments, Dentsply, Gloucester, UK) in a slow-speed WA56A handpiece (W&H Dentalwerk Bürmoos GmbH, Bürmoos, Austria). In this group, no magnification was used by the operator for the whole procedure. The excavation endpoint was verified by using a sharp dental explorer to verify the removal of soft ‘caries-infected’ dentine and retaining a leathery dentine layer (Schwendicke *et al.* 2016). A sample of the remaining deep carious/non-carious dentine after finishing cavity preparation in each tooth was collected with another sterile spoon excavator and transferred into a second pre-weighted Eppendorf tube to be used for the microbiological analysis (see Chapter 6). Operator gloves exchanged with a new pair before the collection of the second sample.

In group B (*“minimally invasive”* or “experimental”), a high-speed handpiece with carbide and diamond burs with copious water jet were used alongside with magnification aid up to 20x provided by a dental operating microscope (G6, Global Surgical Corporation, St. Louis, MO, USA) to gain appropriate access to the EDJ. A similar procedure for the microbiological sampling collection followed. Carisolv™ gel (Rubicon Lifesciences, Gothenburg, Sweden) and its specially designed hand instruments were used for caries excavation of the soft carious dentine mass. The gel is supplied as a twin-syringe two-component system and was auto-mixed in the correct proportions (Static Mixer, RLS Global AB, Gothenburg, Sweden) prior to application on the carious dentine lesion. Drops of the gel were applied on the lesion. After waiting for 30 seconds, Carisolv™ hand instruments of the appropriate size (1-5) were used to abrade away the soft carious tissue. After the gel became cloudy, the cavity was rinsed with water and the procedure repeated until the gel remained clear without any turbulence according to manufacturer instructions. This helped verify the endpoint of the excavation process, leaving caries-affected dentine on the bottom of the cavity. In both groups, residual caries-affected dentine was retained on the cavity pulpal wall, as shown in Figure 5-1.

5.2.4.2 Single-visit restoration

After finishing the cavity preparation in both groups, MTA caps (Acteon, Pierre Rolland, Merignac, France) (Figure 5-2) containing mineral trioxide aggregate powder and distilled water were activated as per manufacturer instructions. MTA paste was applied on the pulpal wall of the prepared cavities (~2 mm) and condensed with a paper point. MTA was left for 5-6 minutes to allow initial setting before applying a layer of glass ionomer cement (GIC) (Fuji IX, GC corporation, Japan), which was placed over the MTA as a protective layer base. A total-etch adhesive (Scotchbond Universal, 3M ESPE, St. Paul, MN, USA) bonding procedure was undertaken following the manufacturer's instructions, before placing a resin composite (N'Durance; Septodont, Louisville, KY, USA) as a definitive restoration.

All procedures were undertaken in a single visit as shown in Figure 5-3 and the patients were blinded to what procedure was used. A standardised clinical and radiographic follow-up was undertaken at 12 months (±2 weeks) (T12), with the clinical and radiographical examiners blinded to which group the examined teeth belonged to. A summary of the materials used is described in Table 5-2.

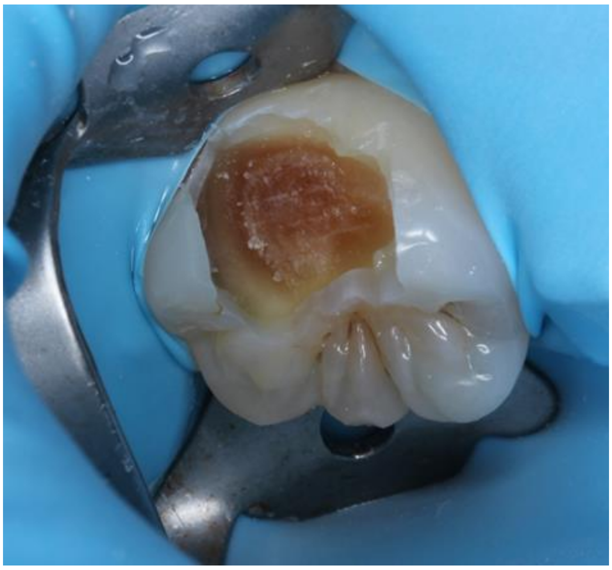


Figure 5-1: Residual caries-affected dentine left on the base of the cavity. In both groups, sound peripheral enamel was preserved to create a good seal for the resin composite restoration.

Figure 5-2: MTA caps (aluminium bag containing 2 x 0.30g capsules)

Table 5-2: Summary of materials used in the trial

Material	Manufacturer
MTA cap: White mineral trioxide aggregate	Acteon, Pierre Rolland, Merignac France
GIC	Fuji IX™ GP, GC corporation, Tokyo, Japan
Resin composite	N'Durance®, Septodont, Cedex, France
Etch and rinse adhesive	Scotchbond Universal, 3M ESPE, St. Paul, MN, USA
Carisolv™ gel and instruments	Rubicon Lifesciences, Gothenburg, Sweden

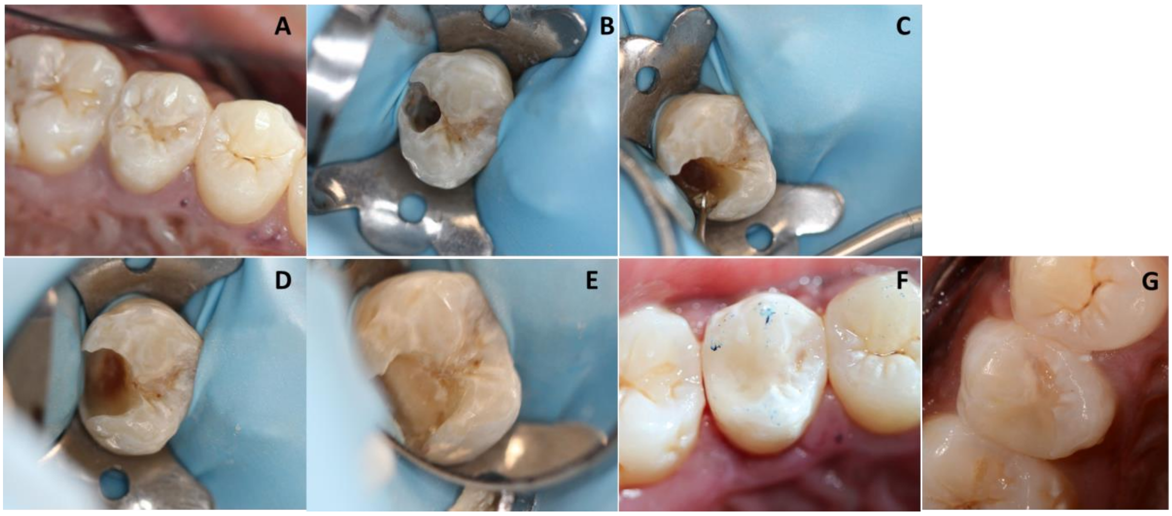


Figure 5-3: Clinical steps of caries excavation in both protocols A) Upper left second premolar with distal deep carious lesion. B) Access to the carious biomass by removing enamel to the level of EDJ. C) Start carious tissue removal according to each protocol to remove caries-infected dentine (soft dentine). D) Leaving a layer of soft carious dentine to prevent pulp exposure. E) Placement of MTA followed by a layer of GIC. F) Final restoration with resin composite in the same visit. G) T12 follow-up.

5.2.5 Radiographic assessment

A digital phosphor plate system (Digora; Optime, Soredex, Tuusula, Finland) was used to obtain periapical radiographs using a paralleling technique with a beam positioning film holder (Dentsply Rinn, Elgin, IL, USA). A dental X-ray machine (Heliodent, Sirona, Bensheim, Germany) operating at 65 kV, 7 mA and an exposure time of 0.16–0.25 s. A small-volume CBCT machine (3D Accuitomo80; J. Morita, Kyoto, Japan) was used to obtain 4 x 4 cm CBCT scans with 0.125 mm resolution, 90 kV, 4 mA and 17.5 s. The accompanying software (i-Dixel 3DX; J. Morita) was used to reconstruct the CBCT data with 1.2 mm slice thickness.

The radiographic penetration depth of the carious lesions was assessed by performing linear measurements on the PA radiographs using ImageJ software (version 1.47, National Institute of Health, USA). The ratio between the full depth of the radiolucent carious lesion and the full dentine thickness was referred to as the penetration depth of carious lesion radiographically. Points A, B and C were placed at EDJ, cavity pulpal wall and roof of pulp chamber respectively. The distance AB represents the full depth of the radiolucent carious lesion, the distance AC represents the full dentine thickness, and the ratio of AB/AC represents the penetration depth of carious lesion as shown in Figure 5-4.

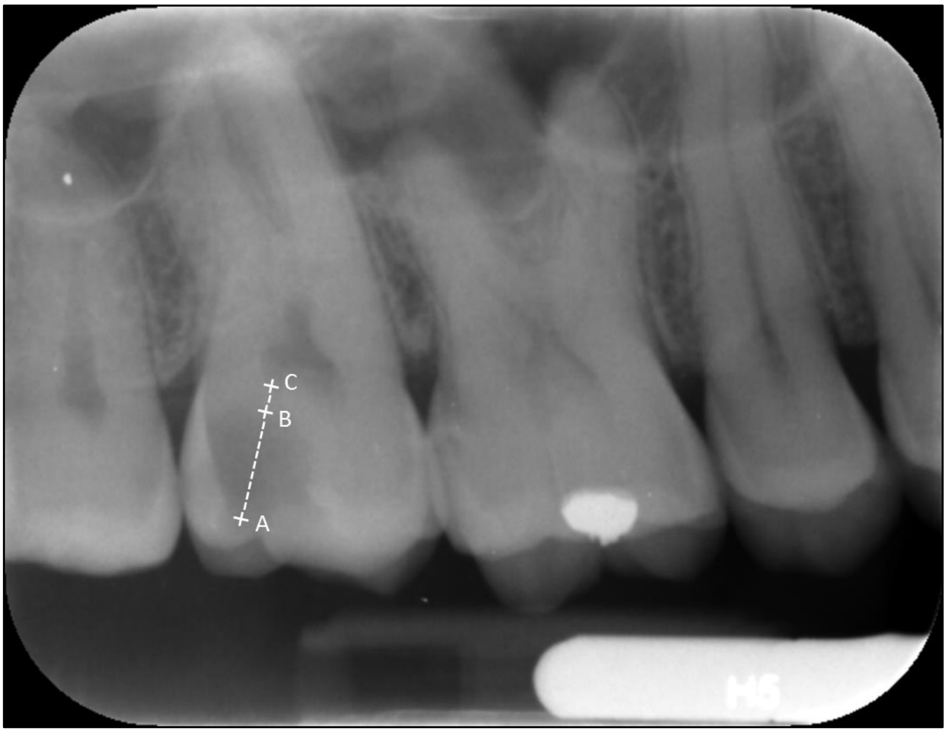


Figure 5-4: Points A, B and C placed at EDJ, cavity pulpal wall and pulp chamber roof respectively. The distance AB represents the full depth of the radiolucent carious lesion, the distance AC represents the full dentine thickness, and the ratio AB/AC represents the penetration depth of carious lesion

In order to obtain CBCT slices that confirmed the presence /absence of periapical radiolucency for each tooth, the dataset was manipulated to adjust slice position in the sagittal, coronal and/or axial planes for each root, as shown in Figure 5-5. The PA and CBCT images as shown in Figure 5-6 were viewed as a PowerPoint presentation (Microsoft, Redmond, WA, USA) on a laptop computer (Pavilion DV6; HP), with a 17-inch backlit LED screen (1280x1024 pixel resolution) in a quiet, dimly-lit room.

An independent consensus panel of two trained, calibrated experienced endodontists with experience in assessing CBCT scans, assessed the T0 and T12 CBCT and PA radiographs jointly. Both examiners were blinded to the operative caries excavation technique used in each evaluated tooth. The reliability of the consensus panel (intra-consensus agreement) was evaluated by together repeating the assessment of the radiographic images after one month. The inter-examiner agreement was evaluated by a randomised assessment of 50% of the PA and CBCT images separately and repeated after one month. Periapical radiolucency referred to the widening of PDL space more than two times the healthy PDL space. An example of a periapical radiolucency is shown in Figure 5-7. The raw data of the CBCT scans were available to the consensus panel, allowing them to examine each CBCT scan in different planes and positions for each tooth using the same computer monitor.

In multirooted teeth, CBCT scan was assessed by each root, therefore a net radiographic decision for that tooth was concluded based on the sum assessment of roots (i.e. if one root has a periapical radiolucency, the tooth was considered to have a periapical radiolucency). The numbering of roots during assessment is illustrated in Table 5-3.

Table 5-3: Root numbering during the radiographical assessment.

Tooth type	Root number		
	1	2	3
Premolars	Single root		
Premolars	Buccal	Lingual/Palatal	
Lower molars	Mesial	Distal	
Maxillary molars	Mesio-buccal	Disto-buccal	Palatal

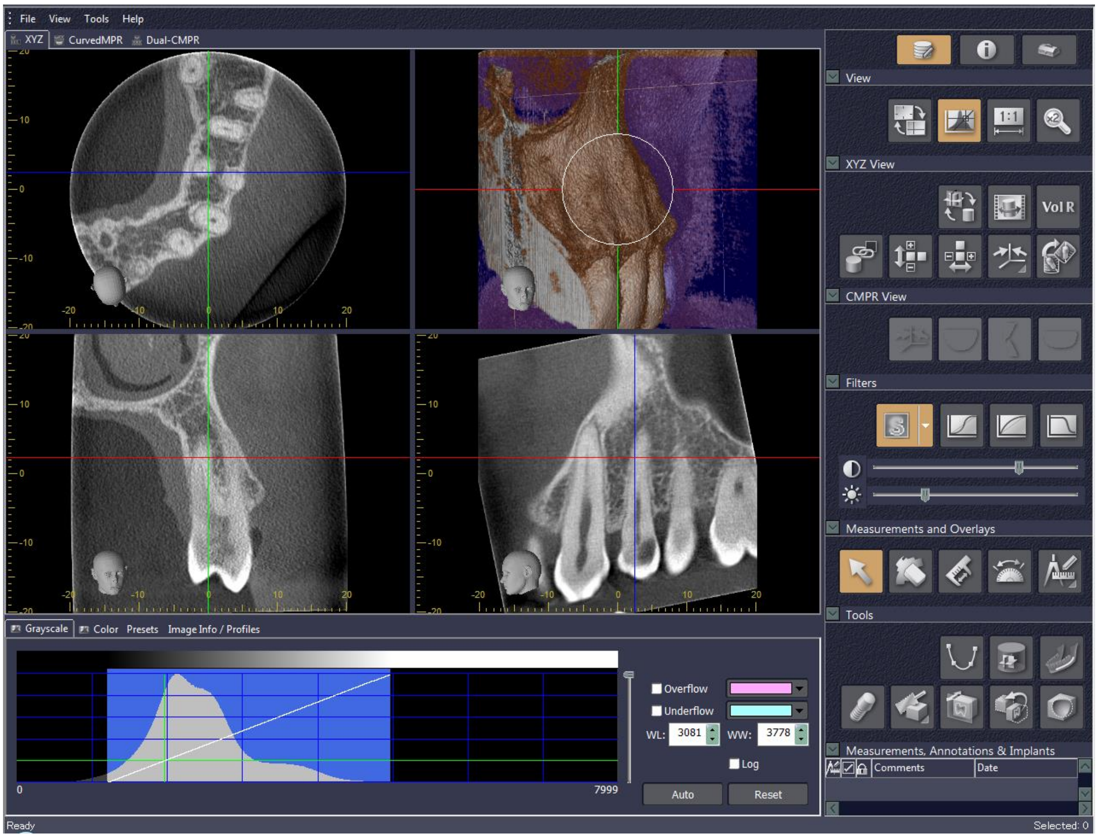


Figure 5-5: Selection of CBCT slice that best confirmed the presence/absence of PA radiolucency in coronal, sagittal and axial planes for each root

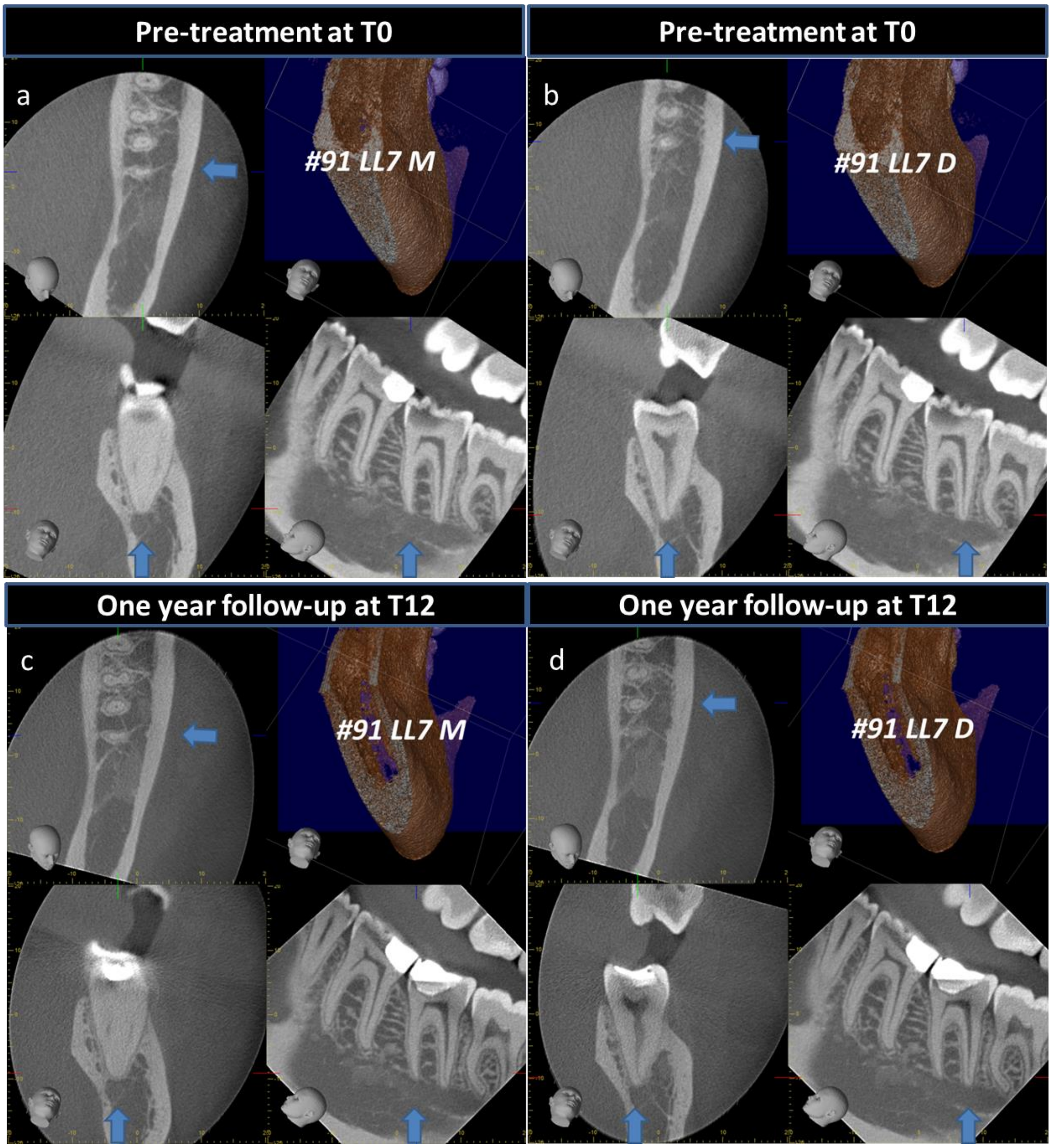


Figure 5-6: CBCT images of each root of the tooth in a PowerPoint presentation/mandibular left second molar. a) and b) show slices of mesial and distal root at T0, c) and d) show slices of mesial and distal roots at T12

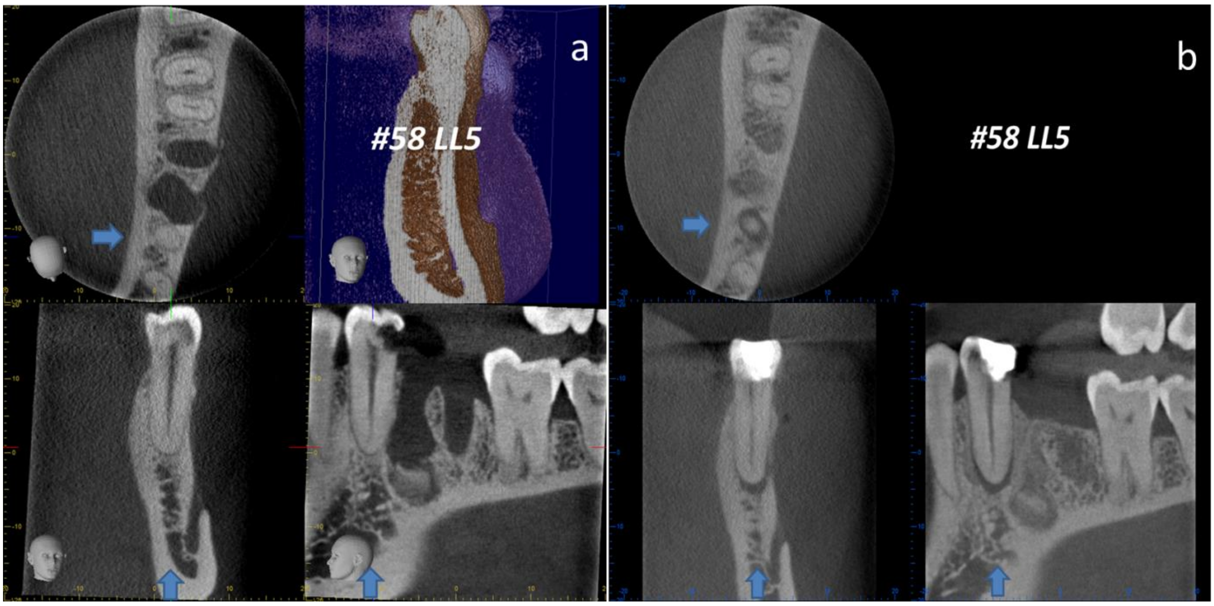


Figure 5-7: Periapical radiolucency in CBCT. a) CBCT slices of a mandibular left second premolar with a healthy PDL space at T0. b) T12 CBCT slices show periapical radiolucency associated with the apex of the tooth

5.2.6 Statistical analysis

The main outcome was the success/failure in each tooth, which was expressed as a binary variable indicating whether the restored tooth maintains/does not maintain its sensibility by combining both clinical and radiographic assessment at T12. Success was determined to be a positive response to cold test and electric pulp testing, the absence of spontaneous pain, no tenderness to percussion, the absence of sinus, fistula and swelling, and absence of PA radiolucency as determined by CBCT scans at T12. The failure was determined to be a negative response to cold and electric pulp testing or presence of spontaneous pain, tenderness to percussion, fistula, sinus, swelling and/or periapical radiolucency in CBCT at T12.

Descriptive statistics were used to summarise various study characteristics and outcome measures. The mean age between the success and failure patients was tested using independent samples t-test. For comparison between groups' clinical outcomes and

PA/CBCT radiographic outcomes, two-tailed two samples z-tests for proportions were used. The association between outcome and other clinical measures such as technique (protocols), the size of the cavity, the intensity of symptoms, gender, age, and type of tooth were assessed using a chi-square test. As the outcome was of binary nature, logistic regression was used to find out the significant predictors of success. In order to increase the power of the analysis, only variables that were significant at liberal 10% level in the above bi-variate analysis were included in the logistic model. For intra-consensus panel agreement and inter-examiner agreement in radiographic assessment, Kappa was used. Calculations were based on the number of teeth. All the analyses were carried out using SPSS version 23.0 and the level of significance was assumed at 5%.

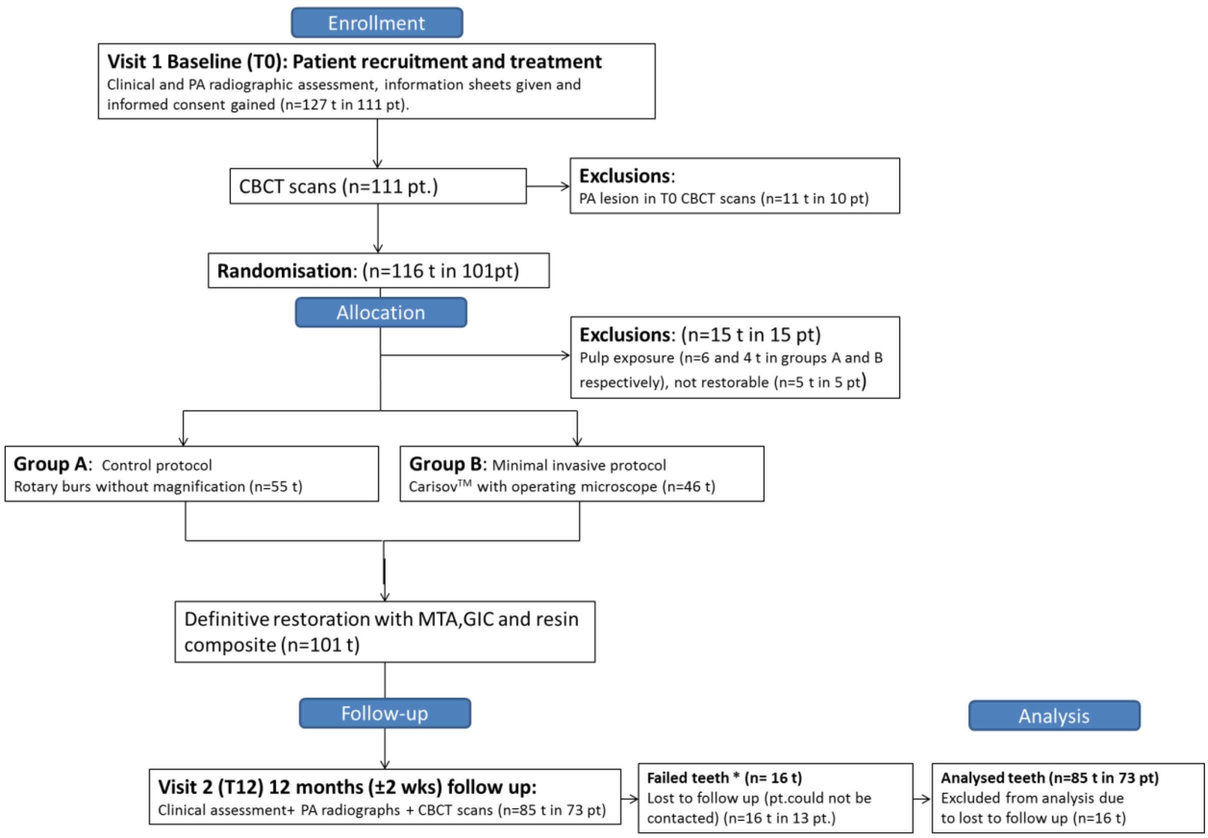


Figure 5-8: Flow diagram indicating patient recruitment and follow-up. Adapted from the CONSORT flow diagram. * Failed teeth are ones which underwent root canal treatments or have a negative response to sensibility tests or have periapical radiolucency in T12 CBCT scans

5.3 Results:

5.3.1 Clinical assessment:

5.3.1.1 Demographic characteristics of teeth in both groups at T0:

A total of 111 patients were recruited, out of which a total of 127 teeth presented with signs and symptoms of reversible pulpitis (using standard clinical and PA radiographs for assessment). 11 teeth in 10 patients showed periapical radiolucencies on the CBCT scans at T0 and were excluded from the study. 10 teeth in 10 patients (six in the control group and four in the minimally invasive group) were excluded from the study because of a pulp exposure during the cavity preparation phase; in addition, five teeth in five patients (three in the control group and two in the minimally invasive group) were excluded because of non-restorability. A CONSORT flow diagram of the patient's recruitments, exclusions and follow-ups are illustrated in Figure 5-8.

101 restorations (55 in the control group and 46 in the minimally invasive group) were placed in the remaining 86 patients. 57/101 (56.9%) and 44/101(43.1%) restorations were placed in females and males respectively (p=0.41). Age of all participants ranged between 19.5-75 yr. (mean age 37.7yr, std=12.2). Recruited teeth were posterior permanent teeth only and the majority of restorations were placed in molars 74/101 (73.3%) compared to premolars, 27/101 (26.7%). Restoration types according to cavity size were distributed according to the following categories in both experimental groups: 9.9% with one wall cavity, 82.1% with two walls and 7.9% with more than two walls.

Distribution of symptoms among the control group was as follows: 43/55 (78.2%) and 12/55 (21.8%) of teeth presented with mild and severe symptoms, respectively. Distribution of symptoms among the minimally invasive group was as follows: 39/46

(84.7%) and 7/46 (15.3%) of teeth presented with mild and severe symptoms, respectively. Demographic characteristics of the included teeth in each group are presented in Table 5-4.

Table 5-4: Distribution of numbers of restorations according to various categories at baseline

Variables	*Control group (A) n=55*	*Minimally invasive (B) group n=46*	*p-value*	*Total in both groups n=101*
Gender				
Males n (%)	*26 (47.2)*	*18 (39.1)*	*0.41*	*44 (43.1)*
Females n (%)	*29 (52.8)*	*28 (60.9)*		*57 (56.9)*
Mean age yr	*37.6 yr*	*37.2 yr*	*0.96*	*37.7 yr*
Tooth type				
Premolars n (%)	*11 (20)*	*16 (34.7)*	*0.09*	*27 (26.7)*
Molars n (%)	*44 (80)*	*30 (65.3)*		*74 (73.3)*
Restorations				
1 wall n (%)	*6 (10.9)*	*4 (8.6)*	*0.71*	*10 (9.9)*
2 walls n (%)	*45 (81.9)*	*38 (82.6)*	*0.92*	*83 (82.1)*
>2 walls n (%)	*4 (7.2)*	*4 (8.6)*	*0.79*	*8 (7.9)*
Symptoms				
Mild n (%)	*43 (78.2)*	*39 (84.7)*	*0.40*	*82 (81.2)*
Severe n (%)	*12 (21.8)*	*7 (15.3)*		*19 (18.8)*

n=number of teeth

5.3.1.2 Clinical outcomes in total and in each group at T12:

At T12, eighty-five teeth (45/control group and 40/minimally invasive group) in 73 patients were recalled for follow-up. Sixteen teeth (10 in the control group and six in the minimally invasive group) in 13 patients were lost to follow-up at T12 because of changes in contact details or because they refused to attend the review appointment. The follow-up rate was 84.1% and 84.8% for teeth and patients, respectively; the total number of teeth reviewed and included in the statistical analysis was 85 teeth in 73 patients, as shown in Figure 5-8.

The clinical success rate (defined as a sustained pulp sensibility with no PA radiolucency in the CBCT scan at T12) in total was 81.2 % (69/85) and the failure rate was 18.8% (16/85) at T12. Of the failed teeth, 10/16 (62.5%) teeth received RCT before the T12 follow-up visit, 5/16 (31.2%) teeth developed periapical radiolucency in CBCT with a positive response to thermal and electrical sensibility tests at T12. One tooth 1/16 (6.2%) developed a PA lesion in CBCT with a negative response to thermal and electric sensibility tests at T12. The total clinical success rates were 87% and 81.2% ($z=1.4$, $p=0.16$) using PA radiography and CBCT scans, respectively.

The success rate for the control group was significantly lower than that of the minimally invasive group (73.3% and 90% in the control and the minimally invasive groups respectively) ($z=1.96$, $p=0.049$). In the control group, 33/45 (73.3%) teeth maintained pulp sensibility with no periapical radiolucency in CBCT at T12, 12/45 (26.7%) teeth failed and were distributed as follows: 8/12 (66%) teeth received RCT before the follow-up visit and 4/12 (33%) teeth developed a periapical radiolucency in CBCT with positive response to pulp sensibility tests at T12. In the minimally invasive group, 36/40 (90%) teeth maintain pulp sensibility with no periapical radiolucency in CBCT at T12, another 4/40 (10%) teeth failed, of which 2/4 (50%) received RCT before the follow-up visit, 1/4 (25%) maintained pulp sensibility but developed a periapical radiolucency in CBCT scan at T12, and 1/4 (25%) developed a periapical radiolucency in CBCT and lost pulp sensibility at T12. Clinical outcomes in both groups are shown in Table 5-5 and Figure 5-9.

The success rates of each group appear to be higher if PA radiographs are used to assess periapical health/pathosis in the follow-ups at T12, with no significant difference between both groups; success rates would be 82.2% and 92.5% (z score= 1.4, p=0.16) in the control and minimally invasive groups, respectively.

Table 5-5: Clinical outcomes after 1 yr follow-up at T12

Outcome (n= analysed teeth)	**Group A (control protocol) (n=45)**	**Group B (minimally invasive protocol) (n=40)**	**95% CI for difference in proportions**	***p*-value**
Success category **Vital pulp without PA radiolucency in CBCT n (%)**	33 (73.3%)	36 (90%)	-0.02 to 0.34	0.049
Failure categories: **Teeth received RCT before recall n (%)**	8 (17.7%)	2 (5%)	0.01 to 0.31	0.07
Vital pulp with PA radiolucency in CBCT n (%)	4 (8.8%)	1 (2.5%)	-0.03 to 0.21	0.21
Non-vital pulp with PA radiolucency in CBCT n (%)	0 (0%)	1 (2.5%)	-0.09 to 0.13	0.28

n=number of teeth

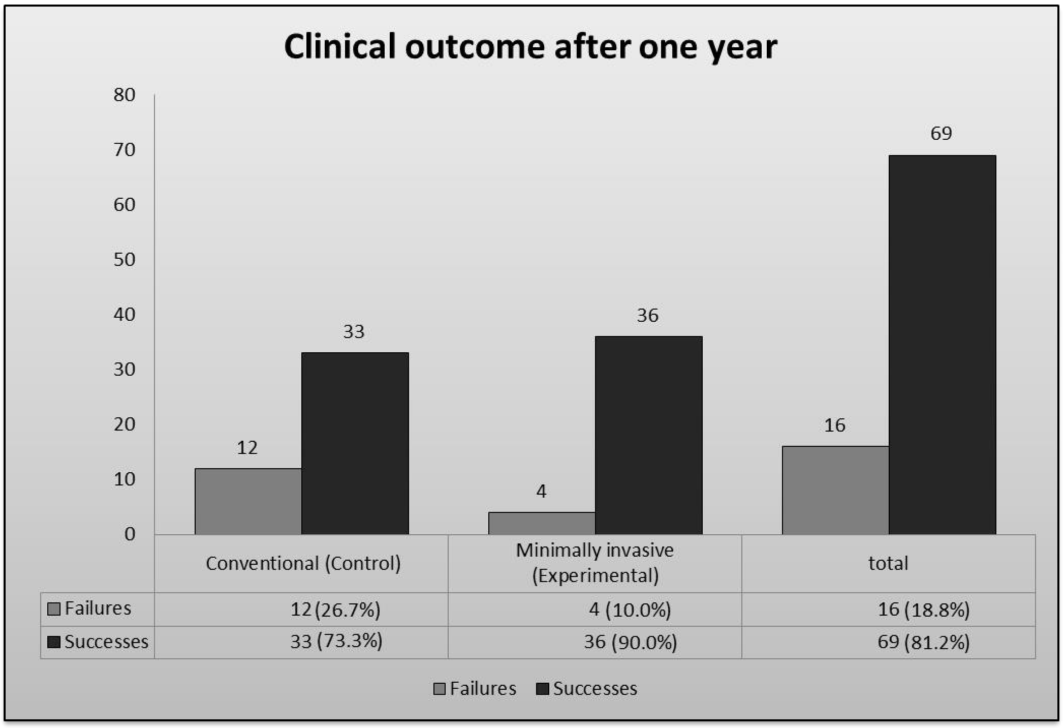


Figure 5-9: Clinical outcome after one year

5.3.1.3 Distribution of the failed teeth at T12 according to T0 symptoms severity

5/11 (45.45%) teeth with severe symptoms failed in the control group, compared to 1/6 (16.6%) teeth with severe symptoms which failed in the minimally invasive group at T12 ($z=1.18$, $p= 0.23$). 7/34 (20.5%) teeth with mild symptoms failed in the control group compared to 3/34 (8.8%) teeth with mild symptoms which failed in the minimally invasive group at T12 ($z=1.36$, $p= 0.17$), as shown in Table 5-6.

In total, 6/17 (35.2%) of teeth with severe symptoms failed at T12 in comparison to 10/68 (14.7%) of teeth with mild symptoms which failed at T12 ($z=1.94$, $p=0.052$) with a borderline level of statistical significance.

Table 5-6: Failure outcome distribution at T12 according to T0 symptoms severity.

Outcome	*Control group n=45*		*Minimally invasive group n=40*		*p-value*	
	Mild n=34	*Severe n=11*	*Mild n=34*	*Severe n=6*	*Mild*	*Severe*
Failure n (%)	7 (20.5%)	5 (45.4%)	3 (8.8%)	1 (16.6%)	0.17	0.23

n=number of teeth

5.3.1.4 Distribution of failed teeth at T12 according to tooth type

4/8 (50%) premolars failed in the control group compared to 3/13 (23%) in the minimally invasive group at T12, with no significant difference (z=1.27, p=0.2) between them.

8/37 (21.6%) molars have failed in the control group compared to 1/27 (3.7%) molars which failed in the minimally invasive group at T12, with statistically significant difference (z=2.03, p=0.042) between them, as shown in Table 5-7.

In total, 7/21 (33.3%) premolars failed compared to 9/64 (14%) molars, with a significantly higher success rate in molars compared to premolars (z=1.96, p=0.049).

Table 5-7: Failure outcome distribution at T12 according to tooth type

Outcome	*Control group n=45*		*Minimally invasive group n=40*		*p-value*	
	premolar n=8	*molar n=37*	*premolar n=13*	*molar n=27*	*premolar*	*molar*
Failure n (%)	4 (50%)	8 (21.6%)	3 (23%)	1 (3.7%)	0.2	0.042

5.3.1.5 Association between clinical measures and clinical outcome at T12

In order to assess if the age of the patients had an association with the outcome, the age of patients was categorised into above and below 40 years old and from the bivariate analysis using chi-square, there was no statistical association between the outcome and the age, gender and cavity size of patients (p=0.98, p=0.36 and p=0.39 respectively). The outcome was significantly associated with techniques (protocols) (p=0.04) only. However, tooth type and symptoms were significant at liberal 10% level (p=0.055 and 0.06 respectively) with the outcome using a chi-square test; these variables were included in the logistic model along with protocols. The results of multivariate logistic regression showed that only techniques and tooth type are the significant predictors of success. When compared to control, the minimally invasive group had 4.42 odds of higher success rates. Similarly, tooth type molar had 4.24 odds of higher success rates when compared to the premolar category. However, the symptoms failed to predict the success rates, as shown in Table 5-8.

Table 5-8: Logistic regression of success significant predictors

Predictor	**Reference**	**Odds ratio**	**95% confidence interval**	***p*-value**
Technique Minimally invasive	Control	4.42	1.12 - 17.45	0.03
Tooth type Molar	Premolar	4.24	1.14 - 15.83	0.03
Symptoms Severe	Mild	0.41	0.11 - 1.48	0.17

5.3.1.6 *Other clinical findings*

Among the excluded teeth, there were 10 teeth excluded because of pulp exposures during the clinical interventions. Six teeth were excavated with the conventional protocol and the other four teeth were excavated with the minimally invasive protocol. Pulp exposure proportions in both protocols were 9.6% (6/61) and 8% (4/50) (z=0.32, p=0.7) of all the teeth that have been excavated in the control and minimally invasive groups, respectively, as shown in Table 5-9.

Table 5-9: Rates of pulp exposures among groups

	Group (control) excavated teeth (n=61)	**Group (minimally invasive) excavated teeth (n=50)**	***p*-value**
Pulp exposure (n= exposed teeth) (% of exposed teeth to the total number of the excavated teeth)	6 (9.8%)	4 (8%)	0.7

It was observed that 15/16 (93.7%) of the failed teeth had two-wall defects, compared to only 1/16 (6.3%) with greater than two walls with defects. Among the failed teeth with two-wall defects, 11/15 (73.3%) teeth were in the control group compared to 4/15 (26.7%) teeth in the minimally invasive group.

Within the groups, 11/37 (29.7%) and 4/32 (12.5%) (z=1.73, p=0.08) of teeth with two wall defects failed in the control and minimally invasive groups, respectively. One failed tooth with greater than two walls defects was in the control group, as shown in Table 5-10.

Clinical assessment of the restorations during the T12 follow-up revealed two restorations in the control group needed to be repaired because of a partial breakdown in the resin composite and none of them was from the failed cases or due to secondary caries.

Table 5-10: Failed teeth number distribution according to wall defect category at T12

	Control group (n=45)	Minimally invasive group (n=40)	Z and p-value
One wall n^* *(%)*	0/5 (0%)	0/4 (0%)	-
Two walls n^* *(%)*	11/37 (29.7%)	4/32 (12.5%)	1.73, 0.08
>2 walls n^* *(%)*	1/3 (33.3%)	0/4 (0%)	2.47, 0.01

*****n=number of failed teeth

5.3.2 Radiographic assessment:

The average ratio of the penetration depth of carious lesions (AB/AC in Figure 5-4) was 78% (Std. ± 7%) of total dentine thickness. Eighty-five and seventy-five (T0+T12) paired CBCT and PA radiographs were analysed (ten teeth were treated with RCT and have no T12 PA/CBCT radiographs).

T0 assessment showed 100% and 98.8% (z=1, p=0.3) of included teeth deemed healthy using PA/CBCT radiographs, respectively, with no significant difference between both radiographic modalities. One included tooth in the minimally invasive group presented with periapical radiolucency at T0 CBCT images, which presented with healed periapical radiolucency at T12 CBCT, as shown in Figure 5-10.

However, in order to assess PA/CBCT detectability, inclusion of the excluded teeth (n=11) that have demonstrated healthy periapical status in the PA radiographs at T0 but showed an initial periapical radiolucency in the CBCT at T0 resulted in 100% and 87%

of teeth deemed healthy using PA/CBCT, respectively, with highly significant difference between both radiographic techniques (z=3.57, p=0.0003) at T0.

The T12 assessment showed that (74/75) of teeth were deemed healthy with PA radiographs, compared to 92% (69/75) (z=1.93, p=0.052) with CBCT scans, with borderline significance, as shown in Table 5-11.

Kappa values for the intra-consensus agreement were 1.00/0.65 for CBCT/PA radiographs respectively and the inter-examiner agreement was 0.64/0.46 for CBCT/PA radiographs, respectively.

Table 5-11: Radiographic assessment results of T0 and T12 PA/CBCT radiographs

Radiographic status	**CBCT**		**PA radiograph**		***p*-value**	
	T0 (n=85)	**T12 (n=75)**	**T0 (n=85)**	**T12 (n=75)**	**T0**	**T12**
Healthy n (%)	84 (98.8%)	69 (92%)	85 (100%)	74 (98.6%)	0.3	0.052

Radiolucency*: PA radiolucency referred to the width of a radiolucency exceed two times the periodontal ligament (PDL) space (Bornstein et al. 2011).

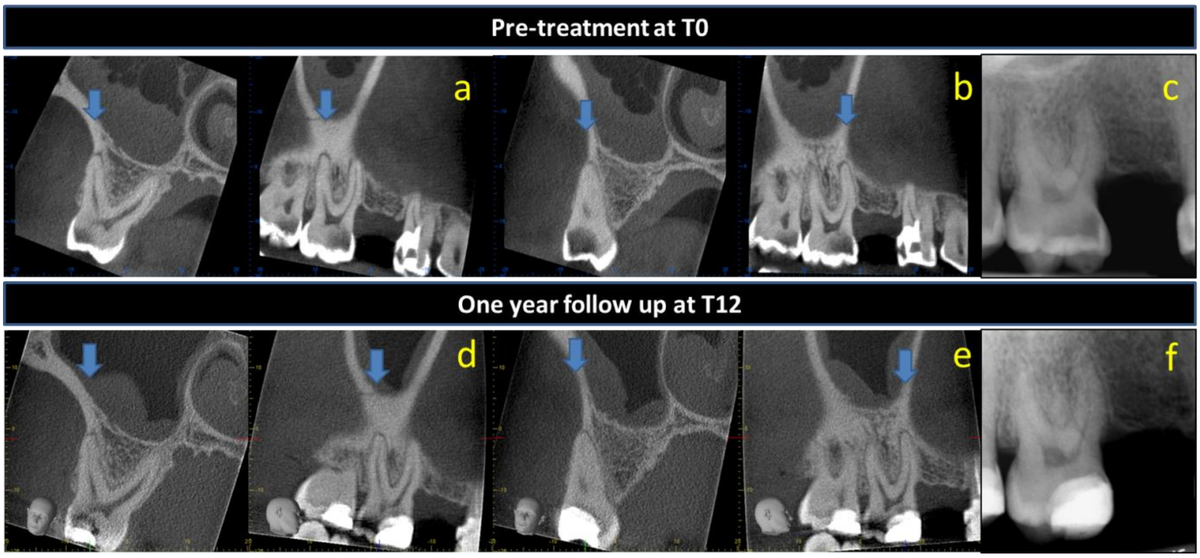


Figure 5-10: Healed radiolucency in upper right first molar: a, b) Coronal and sagittal CBCT images revealing PA lesions in the mesiobuccal and distobuccal roots at T0. c) PA radiograph at T0. d) and e) Coronal and sagittal CBCT images showing healthy PA tissue associated with the mesiobuccal and distobuccal roots at T12. f) PA radiograph at T12

9/75 (12%) of the reviewed teeth showed a partial pulpal calcification radiographically in T12 PA radiographs, as shown in Figure 5-11.

Figure 5-11: Pulp space partial calcification in a lower right second molar after one year of treatment in the control group: a) T0 PA radiograph. b) T12 PA radiograph

5.4 Discussion:

In this RCT, the one-year effectiveness of the minimally invasive protocol has been assessed in comparison to a conventional "control" protocol in the treatment of patients diagnosed with deep caries and reversible pulpitis. It was found that the minimally invasive protocol had a significantly higher success rate compared to the control protocol after one-year follow-up. The biological rationale for the improved response of pulps in teeth treated with the minimally invasive protocol could be the reduced amount of mechanical and thermal irritation caused to the pulp during carious tissue removal. The use of mechanical rotary burs for deep dentine caries removal may be associated with thermal and mechanical irritation to the dentine-pulp complex and non-selective removal of tooth structure (Zach 1972, Yip and Samaranayake 1998, Banerjee *et al.*

2000b, Mjör 2002), which could worsen or cause injury of the dentine-pulp complex by cutting deeper cavity preparations, leaving less remaining dentine thickness which may jeopardise the healing potential of the dental pulp (Stanley 1961, Darwell 1981).

In contrast, previous studies have shown the selective behaviour of the Carisolv™ gel system for caries excavation (Banerjee *et al.* 2000b, Splieth *et al.* 2001). Carisolv™ is less cytotoxic for human dental pulp tissues (Bulut *et al.* 2004) than other chemo-mechanical caries removal agents, such as Papacarie Duo, which shows a rapid slight cytotoxicity and pro-inflammatory action (Garcia-Contreras *et al.* 2014). Carisolv™ gel mixture has a pH of 11; it causes chlorination and cleavage of the denatured collagen fibrils via the chloramines that are formed from interaction between the amino acids and sodium hypochlorite in the gel. These disrupt the collagen cross-linkage in the matrix of carious dentine, by disrupting hydrogen bonding and enhancing removal of unhealthy dentine with hand instruments (Banerjee *et al.* 2000b, Beeley *et al.* 2001, Hamama *et al.* 2014a, Tonami *et al.* 2003).

The selectivity of Carisolv™ gel is attributed to the buffering effect of amino acids in the formula of the gel, which helps in damping the aggressive proteolytic effect of sodium hypochlorite on healthy collagen in caries-affected dentine, thus limiting its effect only to the denatured collagen in caries-infected dentine (Tonami *et al.* 2003). In addition, it has been observed that Carisolv™ gel is able to preserve tooth structure by partially dissolving the denatured collagen without mechanical agitation and also without affecting the sound and demineralised dentine (Hannig 1999).

Clinically, although Carisolv™ gel treatment needed more time than rotary instruments, the chemo-mechanical technique may reduce the need for local anaesthesia and there was no significant difference in clinical efficacy between the Carisolv™ and rotary instruments (Rafique *et al.* 2003, Lai *et al.* 2015). It has been reported that Carisolv^{TM} gel excavation leaves more residual caries than rotary burs (Splieth *et al.* 2001), which may raise concerns regarding restoration integrity and longevity. However, it has been reported that leaving caries under restoration in deep caries treatment has a 3.8% annual failure rate (Schwendicke *et al.* 2013b), compared to 5.7% in the conventional excavation (Lucarotti *et al.* 2005). Also, the arrest of caries progression and remineralisation of remaining caries after partial caries removal has been reported (Bjørndal *et al.* 1997, Maltz *et al.* 2002, Alves *et al.* 2010).

About a third of teeth in patients with severe symptoms were associated with treatment failure, compared to only 14.7% of teeth in patients with mild symptoms with a borderline significant difference between the two treatments. There was statistically no significant association between pre-treatment symptom severity and the clinical outcome after one year. These results disagree with those reported in other studies (Bjørndal *et al.* 2010, Hashem *et al.* 2015) which reported significant associations between pre-operative symptoms intensity and treatment failure. In Bjørndal *et al.* (2010), the presence of pre-operative pain was considered a detrimental factor for good prognosis, as the majority of the recruited cases were pain-free pre-operatively, in contrast to this study which recruited patients with reversible pulpitis. The insignificant association of symptoms intensity with the outcome in this study can be explained by the lower number of included teeth presenting with severe symptoms in both groups and because of the exclusion of patients presenting with CBCT periapical lesions at T0, this might have reduced the probability of a tooth failing, according to symptoms intensity categories. It has been reported that severe symptoms reflect severe histopathosis (Bender 2000b, Aguilar and Linsuwanont 2011). However, symptoms reports in patients with dental pain of pulpal origin are subjective in nature (Levin *et al.* 2009).

Several studies have shown that there is little or no correlation between the histopathologic status of the pulp and the clinical diagnostic findings (Garfunkel *et al.* 1973, Dummer *et al.* 1980, Langeland 1981, Hyman and Cohen 1984). However, these studies did not use CBCT as part of the diagnosis.

Two stepwise excavation studies using clinical symptoms and periapical radiographs for the assessment found a marginal effect of the age of patients on the outcome of the treatment of teeth with deep carious lesions, where patients with younger age showed higher pulpal survival in contrast to the older age patients (Bjørndal 1999, Bjørndal *et al.* 2010) (median age 24 and 29 yr respectively). An influence of age on success/failure of pulp-capped teeth could not be seen in the present study, possibly because of the older age range of participants in this study (mean age 37.7yr). However, this is in agreement with the CBCT study of Hashem *et al.* (2015), who also found no significant association between the outcome and age in IPC.

A proportion of the teeth reviewed radiographically after one year of treatment showed a reduction of the volume of the pulp chamber. There are many factors that could affect the formation of tertiary (reactionary) dentine leading to this radiographic appearance, such as residual dentine thickness (Stanley *et al.* 1983), damage to odontoblast cell processes (Tidmarsh 1981), nerve damage (Okamura *et al.* 1995) and intra-tubular elements damage (Dai *et al.* 1991), presence of growth hormone factors (Sloan and Smith 1999) and placement of MTA as a solubilising agent of growth factors from the dentine matrix (Tomson *et al.* 2007). These may upregulate the generation of tertiary dentine from odontoblast in the dental pulp, in addition to the effect of other dentine changes on pulp activity during restorative treatment and pathological phases of the disease.

In previous retrospective and prospective studies, variable success rates of IPC procedures were reported using different types of liners (Al-Zayer *et al.* 2003, Gruythuysen *et al.* 2010, Farooq *et al.* 2000, Maltz *et al.* 2007, Marchi *et al.* 2007, Orhan *et al.* 2010, Pinto *et al.* 2006). Studies that have used calcium silicate-based materials as IPC liners (Petrou *et al.* 2014, Hashem *et al.* 2015) reported no significant difference between the materials used in the clinical success after six months and one year, respectively. Although no statistically significant difference was detected in the clinical efficacy of Biodentine/GIC when used as indirect pulp capping materials in patients with reversible pulpitis, CBCT showed a significant difference in that most healed CBCT lesions had received Biodentine while most that did not heal received Fuji IX (Hashem *et al.* 2015). It was reported that MTA is more successful than calcium hydroxide in direct pulp capping, as MTA has been shown to induce the recruitment and proliferation of undifferentiated cells to form a dentine bridge, and more predictable hard tissue formation while reducing inflammation compared with calcium hydroxide (Aeinehchi *et al.* 2003, Qudeimat *et al.* 2007, Mente *et al.* 2010, Holland *et al.* 2001, Farsi *et al.* 2007, Nair *et al.* 2008). For this reason, MTA was used in the present study.

Previous studies on caries excavation used standardised periapical radiography to assess the periapical health/pathosis of the teeth (Orhan *et al.* 2010, Bjørndal *et al.* 2010, Maltz *et al.* 2012). However, the ability of periapical radiography to detect apical radiolucencies is limited by anatomical noise and geometrical distortion compared to CBCT (Patel *et al.* 2009, S Patel *et al.* 2012). Using histological standards, CBCT was significantly more sensitive than PA radiography in detecting periapical lesions (de Paula-Silva *et al.* 2009, Kanagasingam *et al.* 2017).

The findings of Hashem *et al.*'s CBCT study, who investigated IPC treatment of teeth with reversible pulpitis and utilised Carisolv^{TM} gel for excavation of carious dentine without magnification, showed that only 65.4% of teeth were deemed healthy using CBCT assessment after one year of treatment (18 teeth found with CBCT radiolucencies out of 52 teeth) (Hashem *et al.* 2015). In comparison to the results of this study, statistically, this is not significantly different from the success rate of the control group (73.3%) ($z=0.91$, $P=0.3$). However, it is significantly lower than the success rate in the minimally invasive group of the present study (90%) ($z=2.74$, $p=0.006$), in which operative microscopes were used in caries removal. The use of the microscope in minimally invasive dentistry increases the visual capacity of the operator and allows for more precise judgment, better control of sensitive procedures and avoids damaging healthy soft and hard tissue (Sitbon *et al.* 2014).

In the CBCT IPC study by Hashem *et al.* (2015), the teeth were restored with a resin composite restoration one month after caries removal and placement of Biodentine or GIC. It has been reported that etched/non-etched Biodentine™ exhibited significant leakage when it was used as a dentine substitute under resin composite in sandwich restorations compared to GIC; also, it exhibits both structural and chemical changes after etching with 37% phosphoric acid in comparison to GIC (Camilleri 2013). In contrast, MTA in this study was layered with GIC before placement of direct resin composite in a single visit in order to avoid the deteriorating effect of etching agents. It was found that the bond between resin composite and etched freshly-mixed MTA was significantly lower than that to fully set MTA (Chapter 3). The placement of good sealing restoration in IPC is important to prevent leakage and arrest the activity of the remaining caries lesion. It has been reported that incomplete caries removal and placement of a definitive restoration in a one-visit treatment of deep caries significantly reduce the annual failure rate (pulpal and non-pulpal) compared to two-step deep caries excavation (Schwendicke *et al.* 2013b).

There was an increase in the failure rates progressively from 0%, 29.7 % and 33.3% in the one-wall, two-walls and > two-walls restorations respectively in the control group compared to the minimally invasive group, which showed 12.5% failures in the two-walls category only, as shown in Table 5-10, which indicates a favourable outcome in the minimally invasive treated teeth. This agrees with a meta-analysis which showed that teeth with multi-surfaces restorations are usually associated with a higher risk of compromised pulps, with a significantly higher annual failure rate compared to the single-surface restorations (Schwendicke *et al.* 2013b). Similarly, it has been reported that long-term pulp necrosis because of restoration failure can be expected when the number of restored surfaces increases (Maltz *et al.* 2012).

Tooth type was a significant predictor of success/failure of the treatment, with molars having higher odds of success compared to premolars. The failure rates in premolars were 50% and 23% in the control and the minimally invasive groups, respectively, with no statistically significant difference between both groups. However, the study was not statistically powered for this comparison. In contrast, molars showed significantly higher success in the minimally invasive group compared to the control group, as shown in Table 5-7. In the treatment of teeth with deep caries and some degree of pulp involvement, new factors can contribute to the higher susceptibility of premolars' pulps to not respond favourably to the treatment compared to molars, such as the mesiodistal dimensions of the cervical region of the crown, which is important in the management of proximal cavities. Pulp size in molars is usually bigger and can provide collateral neuronal and vascular innervations to inflammed areas in the pulp compared to premolars. Although previous studies based on clinical symptoms and periapical radiographs, but not CBCT compared to this study, were inconclusive regarding the effect of tooth type on the outcome, it was found that after direct pulp capping, molars were more successful than premolars (Hørsted *et al.* 1985).

The rate of pulp exposure in this study (9.8% and 8% in the control and the minimally invasive groups respectively) was comparable to the pulp exposure rates (6-8%) reported in another excavation study (Orhan *et al.* 2010). However, it is lower than the rate of pulp exposure in non-selective (complete) caries excavation reported by both Orhan *et al.* (2010) and Bjørndal *et al.* (2010) (22% and 28.9%, respectively). Also, it is lower than the rates of pulp exposures after stepwise excavation (after re-entry) reported by Magnusson and Sundell (1977), Leksell et al. (1996) and Bjørndal et al. (2010) (15%, 18% and 17.5%, respectively). Selective "incomplete" caries removal reduces significantly the risk of pulp exposure and post-operative pulp symptoms (Schwendicke *et al.* 2013a). It was concluded that partial caries removal reduces the incidence of pulp exposure by 77% compared to complete caries removal (Ricketts *et al.* 2013).

CBCT radiographic assessment revealed higher intra-consensus panel agreement compared to PA radiographs because of the superior ability of small-volume CBCT scans to detect small newly-developed PA lesions in teeth with signs and symptoms of inflammation. These results are concurring with other studies that compared CBCT to PA radiographs in detecting PA changes (Patel *et al.* 2009, S. Patel *et al.* 2012, Hashem *et al.* 2015). Moderate inter-examiner agreement in this study was in agreement with other studies that compared CBCT and PA radiography (Pope *et al.* 2014, Hashem *et al.* 2015). Other studies reported lower inter-examiner agreement among experienced examiners (Sogur *et al.* 2009, Tewary *et al.* 2011). In this study, both examiners were specialised in CBCT usage and PA changes detection and assessments sessions were conducted in separate sessions to avoid examiner fatigue.

Pulpal sensibility tests are subjective and indicate neuronal rather than vascular viability (Levin 2013), which provides further support to the use of CBCT when assessing the outcome of IPC in the context of clinical trials (Patel *et al.* 2014).

5.4.1 Conclusions:

1- The one-year effectiveness of the minimally invasive protocol in preserving pulp sensibility of teeth presenting with reversible pulpitis was higher than that of the control protocol, therefore the null hypothesis has been rejected.

2- There were no associations between the one-year outcome and age, gender, symptoms severity and cavity size.

3- The treatment of molar teeth has higher odds of success compared to that in premolars.

4- Symptoms severity indicated a non-significant role in predicting treatment prognosis in patients with reversible pulpitis in this study.

5- CBCT was significantly more reliable in detecting early PA radiolucencies in teeth diagnosed with reversible pulpitis compared to PA radiographs, which resulted in a significant difference between the two protocols, which would not have been detected using periapical radiographs only.

Chapter 6 Bacterial Diversity within Deep Carious Lesions and Correlation to Clinical Presentation, Symptoms and Outcome

6.1 Introduction:

Traditionally, the microbiota present in carious lesions has been investigated using culture-based approaches, utilising various media under aerobic and anaerobic conditions. A large range of different bacterial phyla and taxa have been isolated, predominantly from the genera *Streptococcus, Actinomyces, Lactobacillus, Bifidobacterium, Rothia, Arachnia, Eubacterium, Propionibacterium, Veillonella* and *Prevotella* (Hahn *et al.* 1991, Van Houte 1994). Using modern molecular biology techniques has revealed that only a small proportion of bacteria present can be grown using traditional culture techniques and these non-culturable bacteria account for a large proportion of the organisms present (Wade *et al.* 2016). Methods such as 16S rRNA clonal analysis revealed higher complexity of microbiota in dental caries than was observed by culture and led to the identification of richer genera of bacteria, such as *Streptococcus* (and its *mutans* species, specifically), *non-mutans Streptococcus, Actinomyces, Lactobacillus, Bifidobacterium, Propionibacterium, Veillonella, Selenomonas* and *Atopobium* (Becker *et al.* 2002, Munson *et al.* 2004, Aas *et al.* 2008). Also, it has been noticed that the microflora of secondary caries biofilm was found to include *Prevotella, Veillonella, Lactobacillus, Streptococcus mutans, Neisseriae* and *Actinomyces;* followed by *Peptostreptococcus, Fusobacterium*, and *Porphyromonas gingivalis;* and occasionally *Capnocytophaga* (Mo *et al.* 2010). This bacterial spectrum was found to be similar to that of the microflora in subgingival plaque of periodontal disease and infected root canals (Drucker *et al.* 1991, Hoshino *et al.* 1992).

Dentine caries is associated with a complex microbial ecosystem, subject to change by fluctuations in nutrient concentration, oxygenation and pH. It was found that dentine caries can harbour aciduric bacterial species in different depths of the lesions using solid pH-selective media (Hedenbjörk-Lager and Ericson 2013). Caries-associated microorganisms such as *Streptococcus mutans*, *Streptococcus sobrinus*, and *Lactobacillus* are known to be acidogenic and aciduric. Therefore, they attack enamel and dentine using their acids and compete with other species in an acidic environment, giving them a survival advantage. Also, they are able to penetrate into dentine tubules by binding to collagen type I (Hahn and Liewehr 2007). Nutrient concentration has been found to affect the types of bacterial species present in different depths of the carious lesion. In superficial layers, the presence of carbohydrates and glycoproteins from saliva enhance the growth of gram-positive bacteria such as *Streptococci*. In deeper layers, the proteolytic *Lactobacillus* dominates because of the change in O_2 level and also due to the intake of nutrients which diffuse from the pulp into the deeper layers (Hahn and Liewehr 2007). However, *Lactobacillus* is not always abundant in carious lesions. It was found that carious lesions can be classified microbiologically according to the relative counts of *Lactobacillus* (either high *Streptococcus* or high *Prevotella* lesions) (Munson *et al.* 2004).

Bacterial communities have the ability to adapt to different changes in the environment resulting from different restorative treatments. In a study on primary carious teeth, it was found that the phenotypic heterogeneity increased significantly, from 1.4

phenotypes per *Streptococcus mutans* positive dentine samples at the time of excavation to 2.2 phenotypes after eight weeks. This was attributed to an adaptation of *Streptococcus mutans* to the modified environment under the restoration following caries excavation (Rupf *et al.* 2008). A shift of isolated microbiota (*Lactobacillus spp., Streptococcus mutans, Streptococcus parasanguinis, Actinomyces israelii,* and *Actinomyces gerencseriae*) in sealed carious dentine under restoration after five months of sealing, to a completely different isolated taxa in the microbiota of dentine samples taken after five months which consisted of only *Actinomyces naeslundii, Streptococcus oralis, Streptococcus intermedius,* and *Streptococcus mitis,* was reported. This suggests that the nutrient supply from pulp fluids affected significantly the surviving microbiota, making it less complex compared to that observed in carious lesions open to salivary fluids and pH fluctuations (Paddick *et al.* 2005). Furthermore, it was observed that environmental stresses mostly by pH fluctuations affected the genotypic diversity of *Actinomyces naeslundii* and *Streptococcus oralis* isolated from sound proximal sites in caries-free versus caries-active individuals suggesting that environmental stress may modify a biofilm such that the diversity of the niches is increased.

The excavation process alongside the restorative materials could have an influence on the infectivity and activity of the remaining bacteria in the dentine, by affecting their ecological stress factors like pH, oxygen and nutrients. Different excavation techniques have been proposed for dentinal caries excavation, including rotary mechanical burs, chemomechanical caries removal, laser and air abrasion (M. Marquezan *et al.* 2006, Banerjee 2013, Li *et al.* 2014). Assessing the change in number and phenotypes of bacteria between the front and the base of the lesion after each excavation process is of clinical importance to determine the efficiency of each technique in changing bacteria ecosystem (numbers and phenotypes) in dental caries.

However, bacterial enumeration and identification, especially in the complex microbial communities, by culture-dependent methods is usually time-consuming and often associated with bias and errors, as such bacteria can only grow in selected metabolic and physiologic conditions *in vitro* (Dymock et al., 1996; Kroes et al., 1999). The introduction of non-culture-based techniques has led to the identification of new species of bacteria in the oral cavity. Over 600 oral bacterial species have been identified, one-third of which have not been cultured to date (Nyvad *et al.* 2013, Wade *et al.* 2016). Real-time and quantitative PCR are molecular microbiological techniques developed to enumerate simultaneously and to identify bacterial species according to the design of the primer or the probes used, which are designed around so-called consensus sequences of 16S rRNA gene of specific species which vary little between diverse species (Smith and Osborn 2009). Next-generation high-throughput sequencing has been introduced for non-culture-based approaches for 16S rRNA-based bacterial diversity analyses. This technology allows the detection of low-abundance genera due to a rapid and large number of reads in each sample (Tzanetakis *et al.* 2015). Its low cost and availability led to the growth of the high quality and curated next-generation sequencing datasets (Zaura 2012). Furthermore, the ability to sequence concurrently a large number of samples reduces bias and errors between samples, as the preparation and run conditions can be normalised.

In this study, samples of superficial (subsurface) and inner (deeper, infected/affected dentine interface) carious dentine have been taken from teeth of patients with deep lesions. These lesions have been excavated with the control and minimally invasive protocols mentioned in Chapter 5. DNA was extracted directly from the samples and the 16S rRNA gene was used for bacterial species identification and enumeration and normalised to the mass of sample obtained. The combination of the data will provide a complete picture of the bacteria present and their distribution within and throughout the carious lesion. The overall aim of the bacterial analyses was to determine the phylogenetic range of the organisms recovered from the samples, both inner and outer aspects of the lesions, and to use the data to estimate the change in total species richness of the bacterial community after each excavation process.

6.1.1 Association between Bacteria and Pulpal Inflammation:

It was noticed that the microbiota within carious lesions shifted from facultative gram-positive bacteria to anaerobic gram-positive and negative cocci and rods, during progression into deeper layers of dentine (Hoshino 1985). Also, there was an association shown between the presence of anaerobic gram-negative rods, such as *Porphyromonas*, *Fusobacterium* and *Prevotella* with painful teeth and infected pulps (Massey *et al.* 1993). Similarly, in root canals, anaerobic gram-positive cocci, such as *Peptostreptococci*, have been associated with apical infections (Gomes *et al.* 1996). Research showed that *Micromonas micros* (previously known as *Peptostreptococci*) and *Porphyromonas endodontalis* presence in deep carious lesions was associated significantly with irreversible degeneration of the pulp (Martin *et al.* 2002).

The result of pulp infection depends on the virulence of the attacking microorganisms in caries and the inflammatory response of the pulp to infection (Hahn and Liewehr 2007). It was found that the introduction of certain bacterial components (such as cell wall components of specific bacterial species) into the dentine of human and monkey teeth can elicit pulp inflammation and infiltration of polymorphonuclear leukocytes into the pulp, which is associated with repair (Warfvinge 1985, Warfvinge *et al.* 1985). Also, it has been found that polymorphonuclear leukocytes can infiltrate the pulpal end of the dentine tubules (Bergenholtz 2000).

Different acids produced by bacteria from the fermentation of various nutrients in dental caries can affect the clinical signs and symptoms of carious lesions associated with specific bacterial compositions. Teeth that have carious lesions associated with high counts of *Lactobacillus* usually are not sensitive to thermal stimuli (Hahn *et al.* 1993), because acids (acetic, lactic and propionic acids) produced by these bacteria do not stimulate or suppress the nerve fibres' reaction to thermal stimuli (Panopoulos *et al.* 1983). Teeth with carious lesions that contain *Prevotella*, *Porphyromonas*, and *Fusobacterium* are usually associated with pain because these bacteria produce ammonia, urea and indoles from the fermentation of amino acids and peptides which are nociceptive by-products (Massey *et al.* 1993).

Bacterial endotoxins present in the cell walls of bacteria elicit different inflammatory reactions in the pulp. Symptomatic teeth with irreversible pulpitis have higher levels of endotoxins (those lipopolysaccharides (LPS), present in the cell wall of gram-negative bacteria) in superficial and deep layers of dental caries compared to asymptomatic teeth (Khabbaz *et al.* 2000). Gram-positive bacteria can release lipoteichoic acid (polyglycerol phosphate complex with a glycolipid group) from their cell walls in high concentrations in the low pH conditions found in carious lesions, leading to inflammation of the pulp, as these components cause release of classical inflammatory cytokines including tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-8 (IL-8), interleukin-12 (IL-12) and interleukin-10 (IL-10) (Ginsburg 2002). *Prevotella* bacteria can induce IL-10, which contributes to the elevation of IL-10 mRNA in pulps beneath deep caries (Hahn *et al.* 2000). Also, necrosis of the pulp in rats' teeth was caused by the presence of *Streptococcus mutans* or *Lactobacillus casei* (Paterson and Pountney 1987a, Paterson and Pountney 1987b).

In the present study, samples of superficial and deep carious dentine were collected from patients presenting with signs and symptoms of reversible pulpitis undergoing deep caries excavation undertaken following the two protocols mentioned in Chapter 5.

6.2 Aims

The study aims were to:

1- Determine the change in bacterial load between carious dentine samples taken before and after caries excavation in the teeth of patients presenting with signs and symptoms of reversible pulpitis (as described in Chapter 5) using 16S rRNA non-culture based method for bacterial identification and enumeration.
2- Identify bacterial composition (abundance and prevalence) in superficial and deep dentine caries layers (before versus after excavation).
3- Assess the association between the change in bacterial loads and clinical variables such as symptom intensity, gender, age, the one-year clinical outcome of the treatment and tooth type.
4- Assess the association between prevalence of significant identified bacterial species and clinical variables, such as outcome, symptoms, tooth position, tooth type and age.

6.3 Null Hypotheses:

1- There is no difference between both protocols' effectiveness in changing bacterial loads after excavation.
2- There is no difference in bacterial composition between superficial and deep layers of dentinal caries.
3- There is no association between the bacterial load / composition and clinical variables.

6.4 Materials and Methods:

6.4.1 Patient Sampling

This study was conducted as a part of the randomised clinical trial previously described in Chapter 5. In summary, the carious teeth were isolated with rubber dam to minimise saliva contamination during the excavation procedure; after gaining appropriate access to the dentino-enamel junction and removing the carious enamel, and dental plaque removed with a sterile normal saline flow, the initial samples of superficial carious dentine were collected using a sterile spoon excavator (Ash G5; Claudius Ash Potters Bar, UK) at the beginning of the cavity preparation procedure at a level representing superficial caries-infected dentine. The samples were transferred into sterile pre-weighed Eppendorf tubes marked as “before excavation” to be used for subsequent microbiological analysis. A second sample of remaining carious/non-carious dentine was collected after finishing the cavity preparation using a separate sterile spoon excavator at a level representing “caries-affected dentine” and transferred into another sterile pre-weighted Eppendorf tube marked as “after excavation”, to be used for microbiological analysis (Schulze-Schweifing *et al.* 2014). 106 samples were collected from 53 patients in total (30 treated with the minimally invasive protocol and 23 treated with the control protocol). Clinical interventions, symptoms distribution and one-year outcomes are mentioned in detail in Chapter 5. Each Eppendorf tube was weighed before and after sample collection without further drying of carious / non-carious dentine on a laboratory-grade scale (Mettler-Toledo, AG64, Switzerland) to calculate the weight of the carious / non-carious dentine tissue that had been collected; then 100µl of nucleic acid-free water was added to each sample and these were stored in a -80^{o}C freezer.

6.4.2 DNA Extraction

The DNA extraction procedure was completed following the gram-positive protocol from the manufacturers' instructions (Sigma GenElute Bacterial Genomic DNA Kit). Frozen samples were defrosted and the DNA isolated using Sigma GenElute™ Bacterial Genomic DNA Kits (Sigma-Aldrich, Irvine, UK). 22ml of lysozyme solution was prepared (200µl of lysozyme solution needed for each sample) by adding 22ml of gram-positive lysis solution (L7539) to 4.77 gram of lysozyme (L4919) in order to acquire a lysozyme solution with a concentration of 2.115×10^6 unit/ml. The mixture was dissolved by pipetting. Metal beads and 200µl of the prepared lysozyme solution were added into a disruption tube and subjected to a FastPrep homogenisation for 2x30 seconds at 6.5m/s then incubated at 37^{o}C for 30 min.

20µl of proteinase K solution was added to each sample, followed by 200µl of lysis solution C (B8803). Each tube was vortexed for about 15 seconds and incubated at 55°C for 10 minutes. 500µl of the column preparation solution was added to each pre-assembled GenElute miniprep binding column seated in a 2mL collection tube. The tubes were centrifuged at 12,000 × *g* for two mins and the eluate discarded. 200µL of ethanol (95–100%) were added to the lysate and mixed thoroughly by vortexing for 5–10 seconds, followed by a quick spin for 10 seconds to bring down the beads. The entire contents of the lysate were transferred into the binding column. A wide-bore pipette tip was used to reduce shearing of the DNA when transferring the contents into the column and centrifuged at ≥ 6500 × g for one min. The collection tube containing the eluate was discarded and the column was placed in a new 2mL collection tube.

500µl of wash solution 1 (Sigma-Aldrich, Irvine, UK) was added to a pre-assembled GenElute Miniprep Binding Column (Sigma-Aldrich, Irvine, UK) and centrifuged for one min at ≥ 6500 × g. The collection tube containing the eluate was discarded and the column was replaced with a new 2ml collection tube. A second wash was performed by adding 500µl of wash solution to the column and centrifuged for three min at maximum speed (12,000- 16,000 × *g*) in order to dry the column. Once the cycle ended, the samples were centrifuged for an additional minute at a maximum speed. The collection tubes containing the eluate were discarded and the columns were placed in new tubes. Finally, every DNA sample was eluted adding 50µl of nuclease-free water (Sigma-Aldrich, Irvine, UK), quantified using a NanoDrop fluorescent quantification station and stored at -20^{o}C. An initial quality assurance of the resulting DNA was carried out using a DNA Qbit station to obtain insights into average DNA sizes and integrity.

6.4.3 Total Bacterial Enumeration by Quantitative Polymerase Chain Reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) was performed on all DNA extracts in triplicate to determine the numbers of target 16S rRNA gene. A standard was used which was composed of an extract of standardised dilution of *Pseudomonas aeruginosa* culture (9.43× 10^{+06} copies/ml). The qPCR assays were prepared using a Rotor-Gene Sybr green PCR kit (Qiagen, UK) in a final reaction volume of 20µl containing: 10µl of Rotor-Gene Sybr green "master mix" as supplied by the manufacturer, 8.8µl of RNAse-free water, 0.1µl (concentration of 500nM) of each 16S rRNA gene EubF (5'TCCTACGGGAGGCAGCAGT-3') and EubR (5'-GGACTACCAGGGTATCTAATCCTGTT-3') and 1µl of DNA template (Rogers *et al.* 2013). In the negative control, 1µl of RNAse-free water was used instead of 1µl of DNA template. Cycling settings in the Rotor-Gene Q machine (Qiagen, Crawley, UK) were as follows: initial activation at 95˚C for five min followed by 50 cycles at 95˚C for 15 sec (denaturation) and at 58˚C for 50 sec (combined annealing / extension). A standard curve for the reaction was calculated using Rotor-Gene Q series software (qPCR efficiency = 1.08; R^2 value = 0.995). This was used as the import curve for the other qPCR reactions.

6.4.4 16S rRNA Gene Next-generation Sequencing

The extracted DNAs of 106 clinical specimens collected from 53 patients were processed for 16S rRNA gene sequencing. The samples underwent a quality control check before sequencing, with only 72 samples passing. These 72 samples were prepared for 16S ribosomal RNA gene amplicons according to the Illumina protocol manual (Illumina, 2013). The hypervariable V3 to V5 region was amplified by using 16S Amplicon PCR Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC (Illumina, 2013). Sequencing data acquired for the 72 samples organised according to phylum, class, order, family, genus, and species, after removal of the overhangs, adapters, chimera and outsized products. A QC was carried out on the fastq files obtained using the FastQC tool in Linux, and the libraries compared to the GreenGenes 16S curated database.

6.4.5 Statistical Analysis:

Paired sample t-test was used to compare between bacterial counts before excavation and after excavation. Independent samples t-test was used to compare between the control and the minimally invasive groups in changing bacterial counts. A logarithmic conversion to Log 10 was used to acquire a normal distribution in the bacterial loads. Independent samples Mann-Whitney U test was used to compare the change in the bacterial loads between males and females, premolars and molars, mild and severe symptoms. The Spearman correlation coefficient was used to correlate between age and change in bacterial loads.

Wilcoxon signed rank and McNemar tests were used to compare the abundance and prevalence of bacterial species between superficial and deep dentine respectively. The correlation between the patients' age and the prevalence of each bacterial species (presence or absence) in each superficial or deep carious dentine was determined by using Mann-Whitney U test. Fisher exact test was used to assess the association between bacterial prevalence in both superficial and deep carious dentine and other clinical features. Significance level set in the analysis was 5% (α=0.05).

6.5 Results:

6.5.1 Bacterial Loads from Caries Samples

The mean weight of samples before excavation was 2.9mg ± 2.25, ranging between 0.05 and 8.96mg. The mean weight of samples after excavation was 0.97mg ± 1.18 ranging between 0.04 and 6.1mg, which affected the yield of DNA. Bacterial loads in each sample were expressed as a gene copies number/mg of wet dentine caries before and after excavation, in both groups. This was calculated by converting gene copies number/ml acquired from qPCR runs into gene copies number/mg according to the weight of each sample ((gene copies number/ml x0.1)/ weight in mg).

The bacterial load changes in the samples after excavation exhibited two different trends. Forty-three samples (17 control and 26 minimally invasive groups) showed a reduction in bacterial loads after excavation. However, 10 samples (four and six in the control and minimally invasive groups, respectively) showed higher bacterial loads after excavation compared to bacterial loads before excavation.

The mean gene copies number of the samples before excavation was $3.65 \times 10^{+06}$ gene copy numbers/mg and ranged between ($9.36 \times 10^{+1}$ and $5.15 \times 10^{+7}$) gene copy numbers/mg. The mean gene copy number in the samples after excavation was $4.03 \times 10^{+05}$ gene copy numbers/mg and ranged between ($9.6 \times 10^{+0}$ and $6.3 \times 10^{+6}$) and in the nuclease-free water (no template control) negative control samples was $4.68 \times 10^{+2}$ gene copy numbers/ml, the mean in the positive control was $9.43 \times 10^{+6}$ gene copy numbers/ml.

In total, the mean of reduction (mean reduction = mean gene copy numbers/mg before excavation of all samples – mean gene copy number/mg after excavation of all samples) in all teeth was $3.24 \times 10^{+6}$ gene copy numbers/mg with a reduction percentage equal to 89%. A logarithmic conversion to log 10 was used to normalise the data and a paired sample t-test showed there was a significant difference in the bacterial loads before and after excavation in all samples ($p<0.001$). Bacterial loads and one-year clinical outcome of 53 teeth (106 before and after samples) (21 and 32 patients in the control and the minimally invasive groups) are shown in Figure 6-1 and Table 6-1.

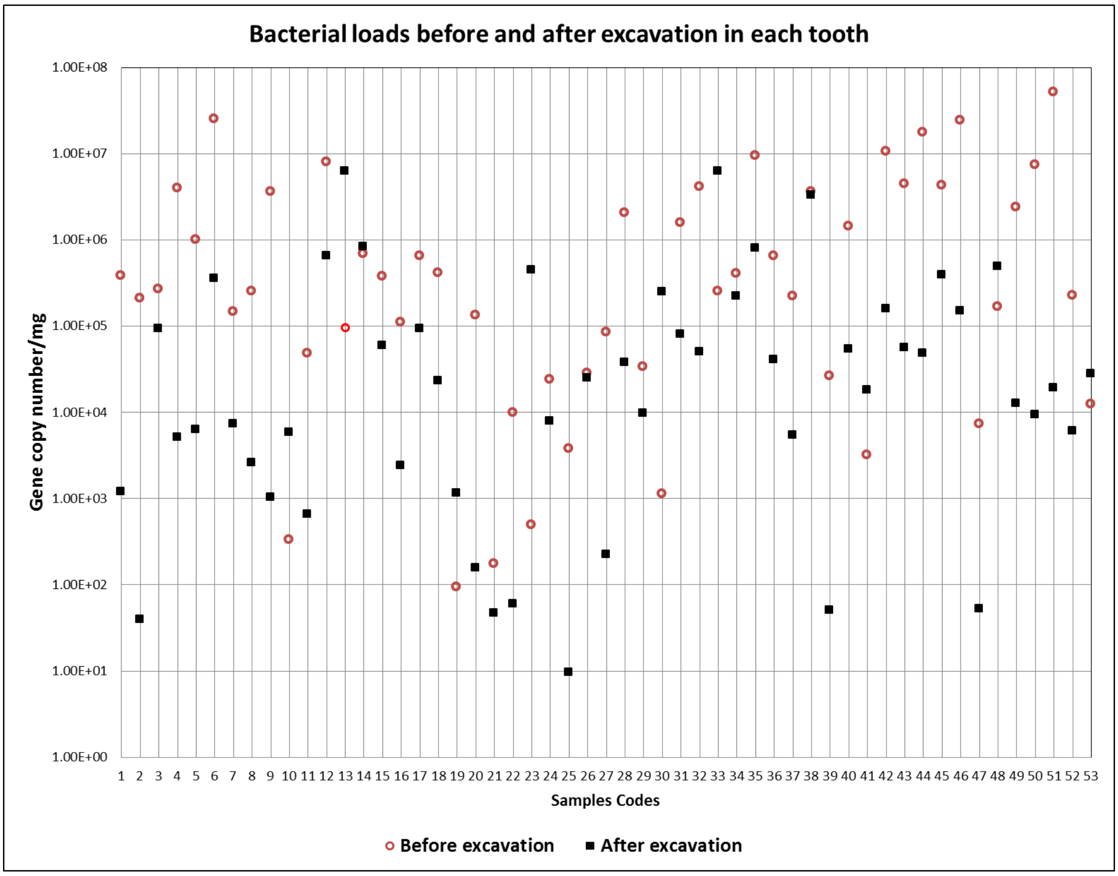


Figure 6-1: Total bacterial loads in samples before and after caries excavation in cavities of 53 teeth with 106 samples before and after excavation in total. Teeth from 1-21 excavated with the control protocol, teeth from 22-53 excavated with the minimally invasive protocol.

Table 6-1: Bacterial load enumeration (gene copy number/mg of dentine) before and after excavation of carious dentine and one-year clinical outcome in the control and the minimally invasive groups.

Control						Minimally invasive					
Patient ID	**Before excavation**	**After excavation**	**Reduction (%)†**	**Signs and Symptoms #**	**Clinical outcome***	**Patient ID**	**Before excavation**	**After excavation**	**Reduction (%)†**	**Signs and Symptoms#**	**Clinical outcome***
1	3.81E+05	1.18E+03	99.7	mild	Success	1	9.93E+03	5.90E+01	99.4	mild	Success
2	2.11E+05	3.94E+01	100.0	mild	lost to follow	2	4.96E+02	4.45E+05	**Increase**	mild	Success
3	2.69E+05	9.39E+04	65.0	mild	failure	3	2.41E+04	7.85E+03	67.4	mild	Success
4	3.98E+06	5.13E+03	99.9	mild	Success	4	3.77E+03	9.61E+00	99.7	mild	Success
5	1.00E+06	6.33E+03	99.4	mild	Success	5	2.82E+04	2.48E+04	11.9	mild	Success
6	2.52E+07	3.56E+05	98.6	mild	Success	6	8.56E+04	2.23E+02	99.7	mild	Success
7	1.46E+05	7.36E+03	95.0	severe	failure	7	2.06E+06	3.74E+04	98.2	mild	Success
8	2.52E+05	2.57E+03	99.0	mild	Success	8	3.38E+04	9.61E+03	71.6	mild	Pulp exposure
9	3.60E+06	1.02E+03	100.0	mild	Success	9	1.13E+03	2.48E+05	**Increase**	mild	Pulp exposure
10	3.34E+02	5.81E+03	**Increase**	severe	Success	10	1.59E+06	7.95E+04	95.0	mild	Pulp exposure
11	4.79E+04	6.53E+02	98.6	severe	Success	11	4.13E+06	4.98E+04	98.8	mild	Success
12	8.01E+06	6.50E+05	91.9	mild	Success	12	2.54E+05	6.23E+06	**Increase**	mild	Success
13	9.71E+04	6.30E+06	**Increase**	severe	Success	13	4.05E+05	2.23E+05	45.0	mild	Success
14	6.84E+05	8.38E+05	**Increase**	mild	Success	14	9.53E+06	7.95E+05	91.7	mild	Lost to follow
15	3.78E+05	5.95E+04	84.3	mild	Failure	15	6.54E+05	4.08E+04	93.8	mild	Success
16	1.10E+05	2.38E+03	97.8	mild	Success	16	2.23E+05	5.37E+03	97.6	mild	Success
17	6.52E+05	9.27E+04	85.8	severe	lost to follow	17	3.65E+06	3.27E+06	10.4	mild	Success
18	4.15E+05	2.33E+04	94.4	mild	lost to follow	18	2.64E+04	5.06E+01	99.8	mild	Lost to follow
19	9.36E+01	1.14E+03	**Increase**	mild	Success	19	1.45E+06	5.39E+04	96.3	mild	Lost to follow
20	1.33E+05	1.55E+02	99.9	mild	Success	20	3.16E+03	1.80E+04	**Increase**	mild	Success
21	1.75E+02	4.69E+01	73.2	mild	Success	21	1.07E+07	1.59E+05	98.5	mild	Success
						22	4.48E+06	5.59E+04	98.8	mild	Success
						23	1.78E+07	4.80E+04	99.7	sever	Success
						24	4.28E+06	3.91E+05	90.9	mild	Success
						25	2.45E+07	1.51E+05	99.4	severe	Lost to follow
						26	7.37E+03	5.22E+01	99.3	Mild	Success
						27	1.67E+05	4.89E+05	**Increase**	mild	Success

						28	2.38E+06	1.27E+04	99.5	mild	Failure
						29	7.40E+06	9.42E+03	99.9	mild	Failure
						30	5.15E+07	1.90E+04	100.0	mild	Success
						31	2.25E+05	6.02E+03	97.3	Severe	Success
						32	1.23E+04	2.77E+04	**Increase**	mild	Success

Signs and symptoms at baseline visit, *Clinical outcome (the one-year clinical outcome of the treatment for each patient) and † percentage of reduction in bacterial loads in samples after excavation compared to that in samples after excavation.

6.5.1.1 Cavities with Higher Bacterial Loads after Caries Excavation:

Ten cavities presented with higher bacterial counts in samples after the excavation process (deep carious dentine) in both groups by a mean of 1.34×10^6 gene copy number/mg with a percentage (percentage of increase = mean increase / mean gene copy number after excavation × 100) equal to 91.6% after excavation. These samples derived from six teeth from the MI protocol and four teeth excavated with the control protocol. After the minimally invasive protocol, the mean of increase in bacterial loads was $1.73 \times 10^{+5}$ gene copy number/mg with the rate of increase equal to 94.1%. After the control protocol, mean of increase in bacterial counts was $1.59 \times 10^{+6}$ gene copy number/mg with a percentage of increase equal to 89.1%. As the data of the bacterial load increase in each group were not normally distributed, a logarithmic conversion to log 10 was used and an independent sample t-test was used to compare between groups. There was no significant difference in the bacterial load increase between the two groups after caries excavation, as shown in Table 6-2.

Table 6-2: The mean and percentage of bacterial loads increase in 10 teeth that exhibited higher bacterial count in the samples after excavation, either with the minimally invasive protocol or the control protocol.

Protocol	Mean of increase in bacterial loads	Percentage of increase	*p*-value*
Minimally invasive protocol	$1.73 \times 10^{+5}$ gene copy number/mg	94.1%	0.49
Control protocol	$1.59 \times 10^{+6}$ gene copy number/mg	89.1%	

* Independent sample t-test

6.5.1.2 Cavities with Lower Bacterial Loads after Caries Excavation:

In 43 teeth, 26 teeth excavated with the minimally invasive protocol and 17 teeth excavated with the control protocol, there was a mean of reduction in the bacterial loads after excavation equal to $4.31 \times 10^{+6}$ with a percentage reduction of 96.5%. In the minimally invasive group, there was a mean of reduction in the bacterial loads equal to

$5.45 \times 10^{+6}$ gene copy number/mg with a reduction percentage equal to 96.3%, while the control protocol was able to reduce bacterial loads in the carious dentine samples by a mean of $2.56 \times 10^{+6}$ gene copy number/mg with a reduction percentage equal to 97.1%. A paired sample t-test after logarithmic normalisation of data shows a significant reduction in bacterial count after excavation ($p<0.001$ and $p=0.001$) in the minimally invasive and control groups, respectively. An independent sample t-test was used to compare between the two protocols and showed there was no significant difference in reduction of the bacterial loads between the control and the minimally invasive groups after caries excavation, as shown in Table 6-3.

Table 6-3: The mean and percentage of bacterial counts reduction in 43 teeth (26 and 17 in the minimally invasive and the control groups respectively) that exhibited a reduction in bacterial counts in dentine samples.

	Mean of reduction in bacterial loads	Percentage of reduction	*p*-value*
Minimally invasive protocol	5.83 × 10+6 gene copy number/mg	96.5%	0.33
Control protocol	3.18 × 10+6 gene copy number/mg	97.1%	

* Independent samples t-test

In total, by using Mann-Whitney u-test for non-parametric data (as the bacterial load difference contained negative values of the teeth that exhibited higher numbers of bacteria in the deep dentine compared to the superficial carious dentine) there was no significant difference between both protocols in changing bacterial loads ($p=0.72$) as shown in Table 6-4.

Table 6-4: The mean and percentage of bacterial counts reduction in 53 teeth (32 and 21 in the minimally invasive and the control groups respectively)

	Mean of reduction in bacterial loads	Percentage of reduction	*p*-value*
Minimally invasive protocol	$4.21 \times 10^{+6}$ gene copy number/mg	91.3%	0.72
Control protocol	1.77 × 10+6 gene copy number/mg	81.5%	

* Independent samples Mann Whitney U-test

6.5.2 Association between Bacterial Load and Clinical Variables

Clinical features including baseline symptom intensity, clinical outcome, age, gender, tooth type and cavity wall involvement of the sampled teeth in this study are shown in Figure 6-2.

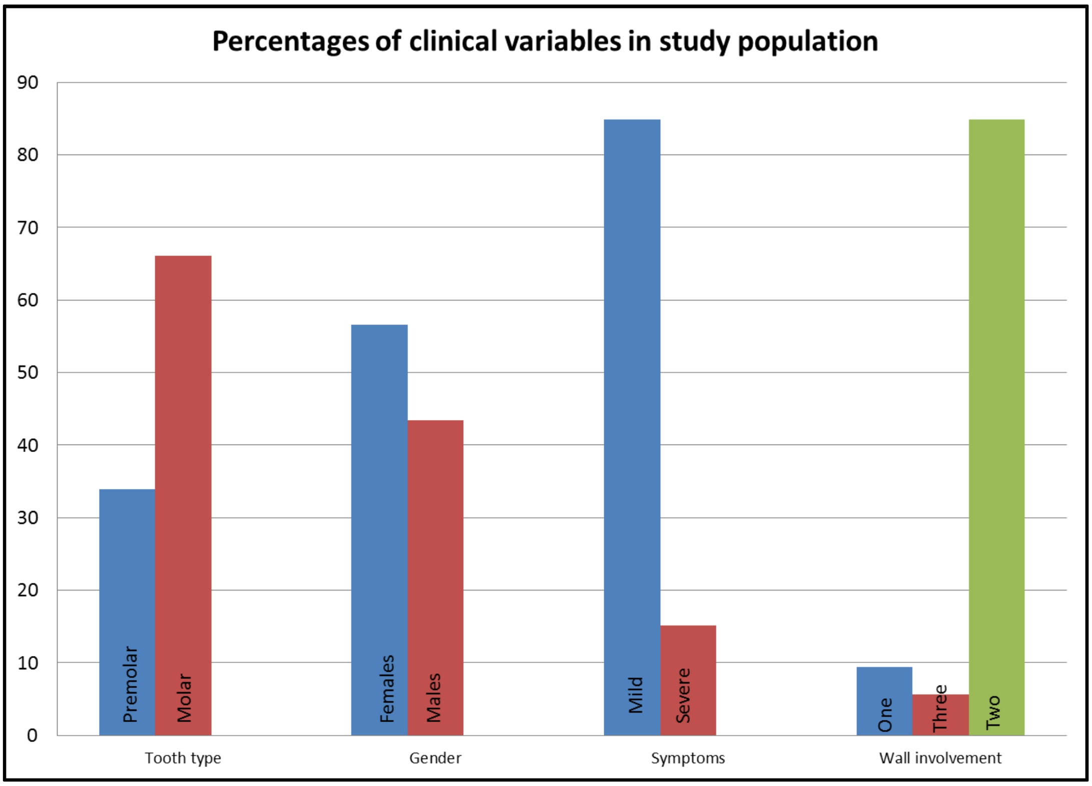


Figure 6-2: Demographic characteristics of study population presented in percentages.

After one year, the success/failure of the treatment was assessed as described in Chapter 5. In the control group, 15 teeth maintained vitality and three failed after one year of treatment; in the minimally invasive group, 23 teeth maintained vitality and two teeth failed after one year, as shown in

Table 6-5.

Table 6-5: The one-year follow-up of 53 teeth treated in the control and the minimally invasive groups

Protocol	Lost to follow *n*	Pulp exposure *n*	Success *n*	Failure *n*
Control	3	0	15	3
Minimally invasive	4	3	23	2

n= number of teeth.

Mann-Whitney u-test was used to compare the bacterial load change between teeth with mild and severe symptoms and between males and females. There was no significant difference in bacterial load change between teeth with mild and severe symptoms (p=0.98) or between teeth of males and females (p=0.72). However, there was a significant difference between molars and premolars in terms of change in bacterial loads before and after excavation (p= 0.036). The extent of reduction of bacteria was higher in premolars compared to molars (percentages of bacterial loads reduction were 96.7% and 84.6% in premolars and molars, respectively). However, after excluding teeth that exhibited higher numbers of bacteria in deep dentine samples compared to superficial dentine samples (two premolars and six molars) from the analysis, there was no significant difference between premolars and molars in terms of bacterial load reduction (p=0.065) as well as between males and females or between mild and severe symptoms (p>0.05 respectively). Similarly, Mann-Whitney u-test showed no significant difference between the failed and successful teeth in terms of bacterial load change (p>0.05). There was a positive but non-significant correlation between age of the patients and the change in bacterial loads using Spearman correlation coefficient (r=0.112, p=0.42).

6.5.3 Microbial Findings of 16S rRNA Sequencing:

The total number of reads acquired from the 72 sequenced samples was 38,548,653 reads; average reads per sample was 741,320.25; minimum reads/sample was 476,747 and the maximum was 995,516. The average percentage of reads that were classified into the genus level was 96.74%; minimum percentage/sample was 89.50% and the maximum percentage/sample was 99.75%.

The average number of species in each sample was 408.21. The average of Shannon Species Diversity index (the measure of species diversity in a given community showing community composition and taking into account the relative abundance of species that are present in the community) for all samples was 2.262. Minimum species number was 225/sample and maximum species number was 671/sample. Superficial carious dentine samples contained 398.2 species/sample compared to 425.5 species/sample in deep carious dentine samples.

6.5.3.1 Phylum Level

The total number of operational taxonomic units (OTUs) that were identified to species level in all samples was 1518. These OTUs belonged to 27 phyla, 50 classes, 102 orders, 221 families and 598 genera. The most abundant five phyla were *Firmicutes,* *Actinobacteria, Bacteriodetes, Proteobacteria, Fusobacteria* and *Spirochaetes*, as shown in Figure 6-3.

Additional phyla were identified such as *Synergistetes, Cyanobacteria, Tenericutes* and *Thermotogae* and others but were less than 0.5% abundance each.

By using paired sample t-test, non-significant differences were found in the abundance of each phylum between the superficial and the deep dentinal caries samples ($p>0.05$).

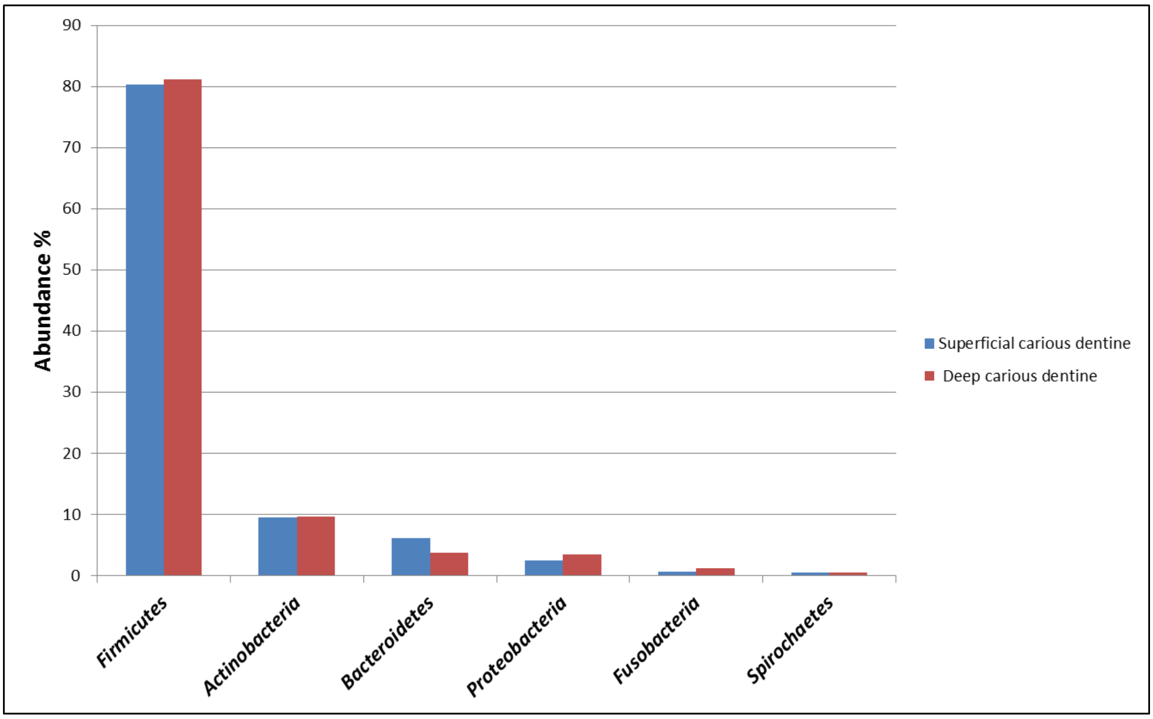


Figure 6-3: Abundance of the most abundant phyla (>0.5 % relative abundance) in superficial and deep carious dentine samples.

The prevalence of the most abundant phyla in superficial and deep carious dentine samples are presented in Figure 6-4. Wilcoxon rank test was used to compare the prevalence of phyla between superficial and deep carious dentine; the results show that there was no significant difference ($p>0.05$) in the prevalence of each phylum between superficial and deep dentinal samples.

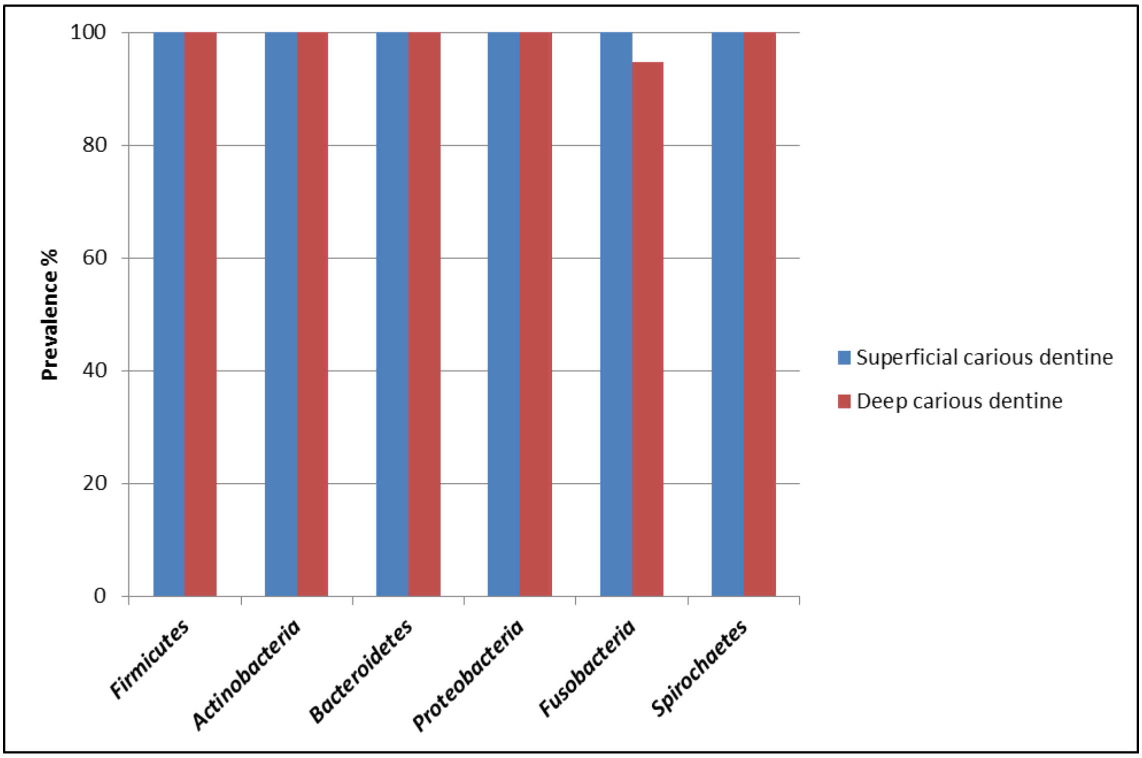


Figure 6-4: Prevalence of top observed phyla in superficial and deep carious dentinal samples.

The top representative classes according to proportion of sequences (>1%) in all samples were *Bacilli, Clostridia*, *Actinobacteria*, *Bacteroidia* and *Betaproteobacteria*, as shown in Figure 6-5.

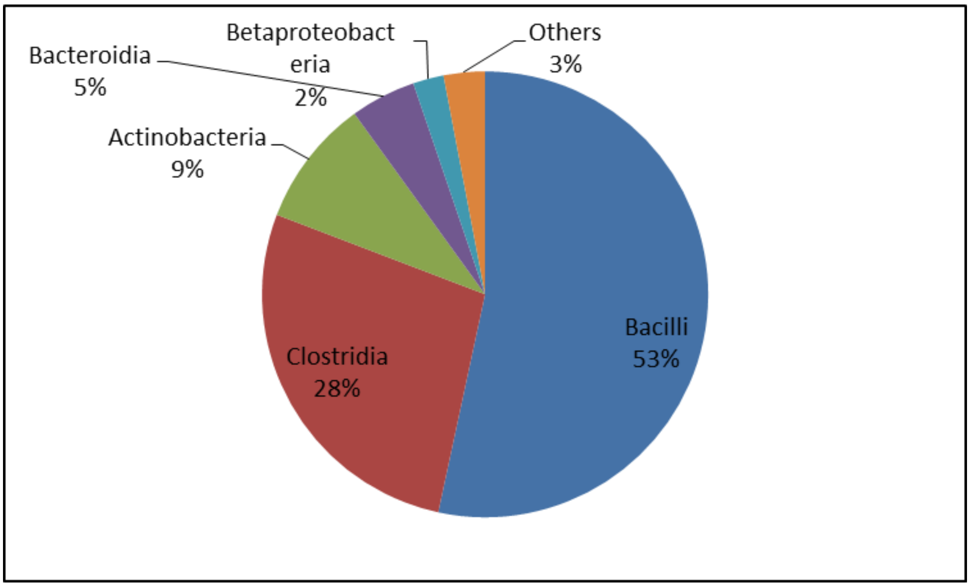


Figure 6-5: Top representative classes according to proportions of sequences in each class. "Others" represent the sum of percentages of the classes below 1% proportion of sequences.

The top representative families according to the proportion of sequences were *Lactobacillaceae, Coriobacteriaceae, Streptococcaceae, Veillonellaceae, Eubacteriaceae* and *Prevotellaceae,* as shown in Figure 6-6.

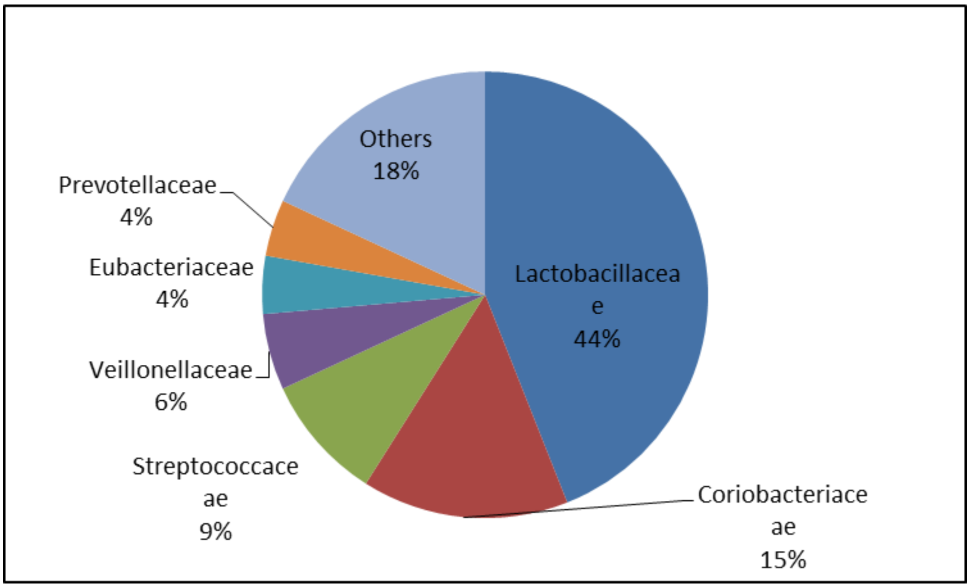


Figure 6-6: Top representative families according to proportions of sequences in each family. "Others" represent the sum of the proportion of sequences of families that have a proportion of sequences below 4%.

6.5.3.2 Genus Level

The sequences belonged to 598 genera. The number of genera that have relative abundance above 0.01% was 94. The average number of genera that have relative abundance above 0.01% per sample was 81.5 ± 7.9. Their averages in superficial and deep carious dentine samples were 82.4 ± 7.5 and 79.7 ± 7.9 genera/sample, respectively.

The top ten abundant genera were *Lactobacillus* (34.6% and 32.1% in superficial and deep carious dentine, respectively), *Atopobium* (12.4 % and 9.4 % in superficial and deep carious dentine, respectively), *Streptococcus* (9.4 % and 9.9 % in superficial and deep carious dentine samples, respectively), *Prevotella*, *Pseudoramibacter*, *Parascardovia*, *Luteococcus*, *Veillonella*,, *Rothia* and *Neisseria*, as shown in Figure 6-7.

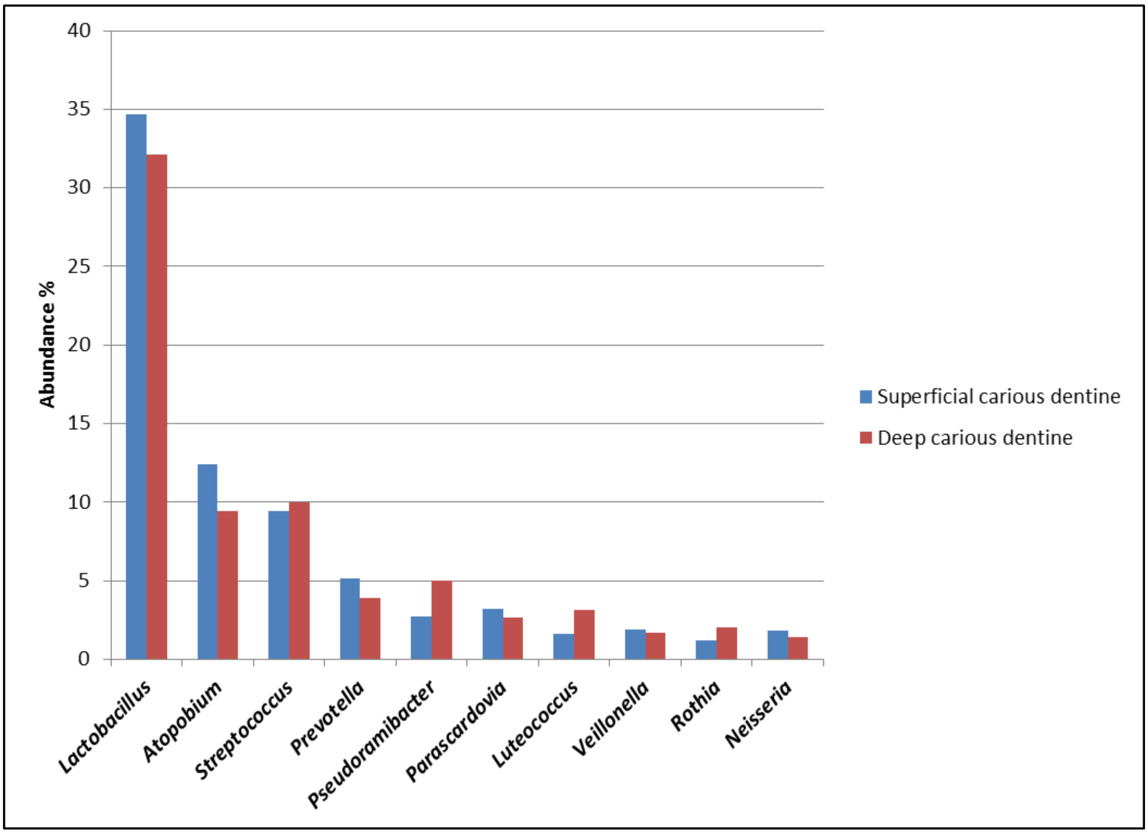


Figure 6-7: Top 10 abundant genera in superficial and deep carious dentine.

Figure 6-8 shows the genera that have the greatest effect size of the difference between abundance (%) in superficial and deep carious dentine.

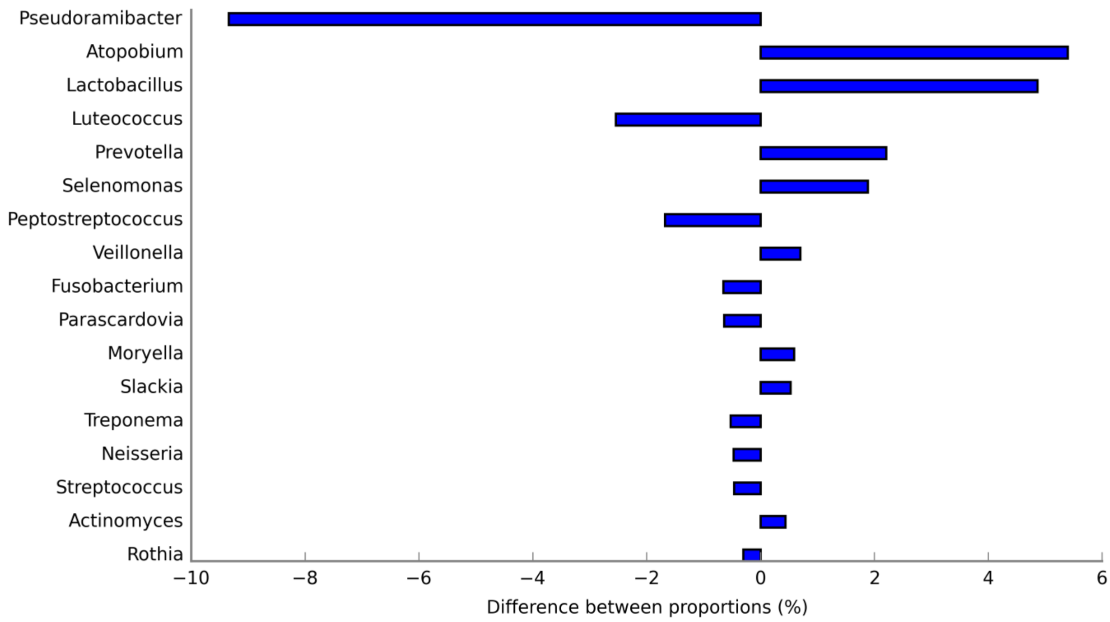


Figure 6-8: Bar plot showing the genera that have the highest effect size of the difference between proportions of superficial and deep carious dentine.

The average abundance of most genera in all samples was below 1%; only 16 and 15 of them were above 1% in superficial and deep carious dentine, respectively. The top ten prevalent genera in superficial and deep carious dentine were *Streptococcus, Prevotella, Atopobium, Veillonella, Actinomyces, Rothia, Fusobacterium, Lactobacillus, Porphyromonas*, *Parascardovia* and *Neisseria*, as shown in Figure 6-9.

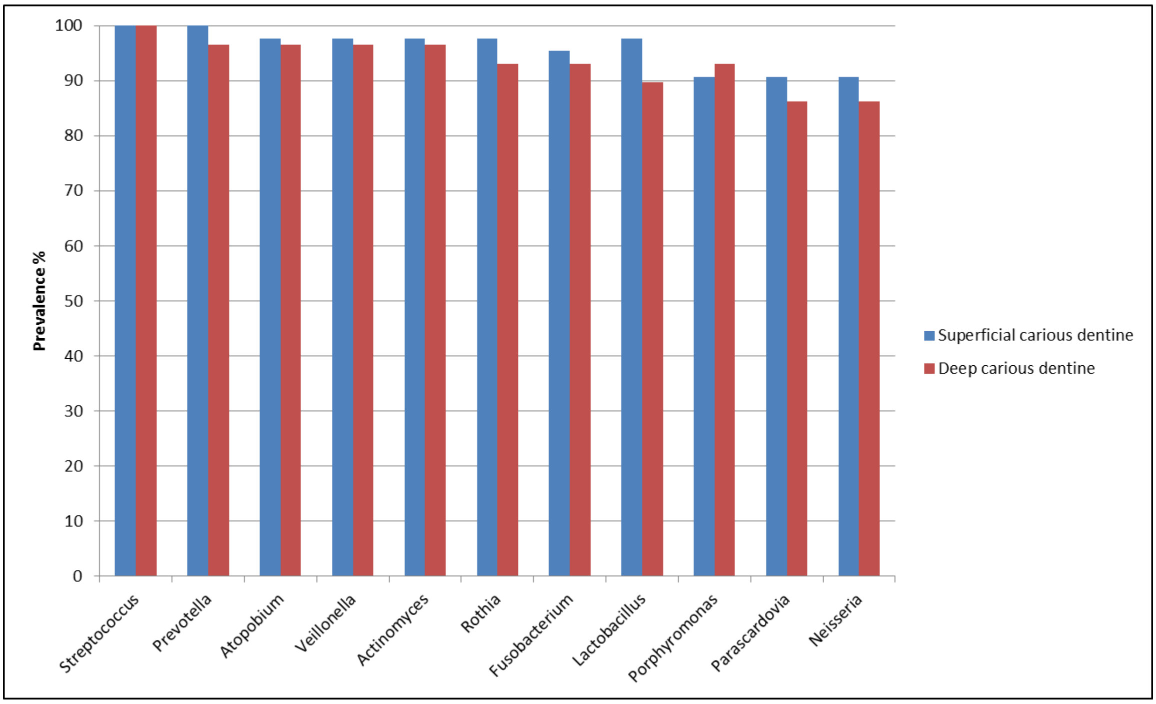


Figure 6-9: Top ten prevalent genera in superficial and deep carious dentine.

Some genera were present in deep carious lesions but not in superficial carious dentine such as *Kutzneria*, *Nocardioides* and *Acidisoma*, with prevalences of 31%, 15% and 15%, respectively. Other genera were detected in superficial but not in deep carious lesions such as *Dethiobacter*, *Klebsiella* and *Agrobacterium*; prevalence being 12%, 9% and 9%, respectively.

Heat-maps of the top 20 abundant genera in superficial and deep carious dentine samples were created to assess the clustering of genera. Results show that the samples can be clustered into three different clusters according to the abundance of *Lactobacillus* in the samples into high-*Lactobacillus*, medium-*Lactobacillus* and low-*Lactobacillus* clusters, as shown in Figure 6-10 and Figure 6-11.

In superficial carious dentine samples (43 samples), high-*Lactobacillus* cluster (85-99% relative abundance) in sample 1-8 was characterised by a low abundance of other genera. Medium-*Lactobacillus* cluster (30-75% relative abundance) in samples 9-22 was characterised by the presence of high abundances of *Atopobium* and *Streptococcus*. Bacterial composition in the low-*Lactobacillus* cluster (0-11% relative abundance) was dominated by *Streptococcus, Atopobium, Prevotella, Parascardovia, Pseudoramibacter*, *Neisseria* and *Peptostreptococcus*, as shown in Figure 6-10. Similarly, in deep carious dentine samples, high-*Lactobacillus* cluster (85-97% relative abundance) was composed mainly of *Lactobacillus* with very low proportions of other bacteria. Medium-*Lactobacillus* cluster (35-63% relative abundance) was also dominated by *Lactobacillus* but associated with high proportions of *Atopobium, Streptococcus* and *Peptostreptococcus*. The last low-*Lactobacillus* cluster (0-17% relative abundance) in deep carious dentine was dominated by *Atopobium, Streptococcus, Pseudoramibacter, Prevotella* and *Luteococcus*, as shown in Figure 6-11.

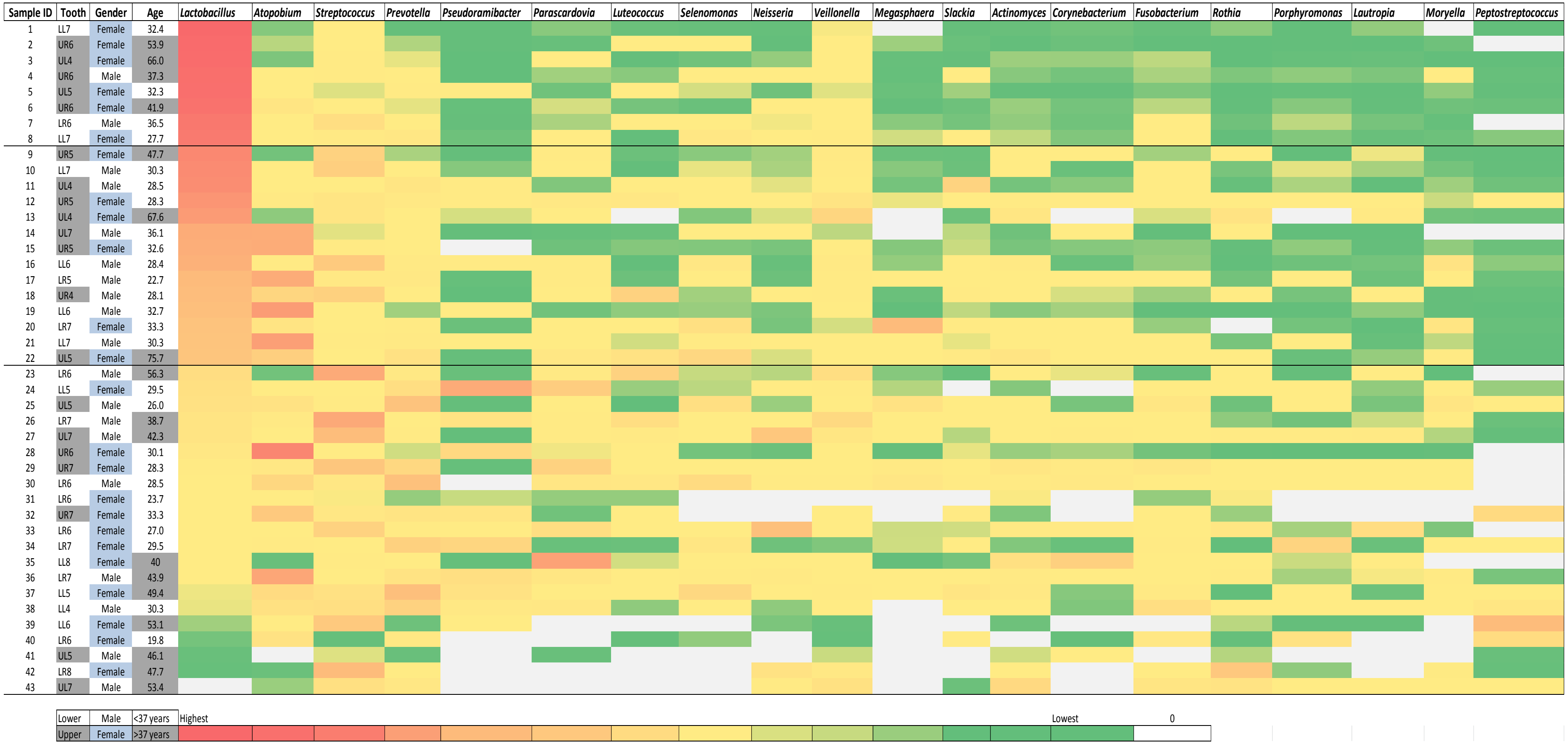


Figure 6-10: Heat-map of top most abundant 20 genera in superficial carious dentine samples (43 samples) divided into three clusters according to the relative abundance of *Lactobacillus*. High-*Lactobacillus* group from samples 1-8 (85-99% relative abundance), medium-*Lactobacillus* group from sample 9-22 (30-75% relative abundance) and low-*Lactobacillus* group from sample 23-42 (0.1-11%). Age, gender and tooth type are indicated on the left side of the heat-map.

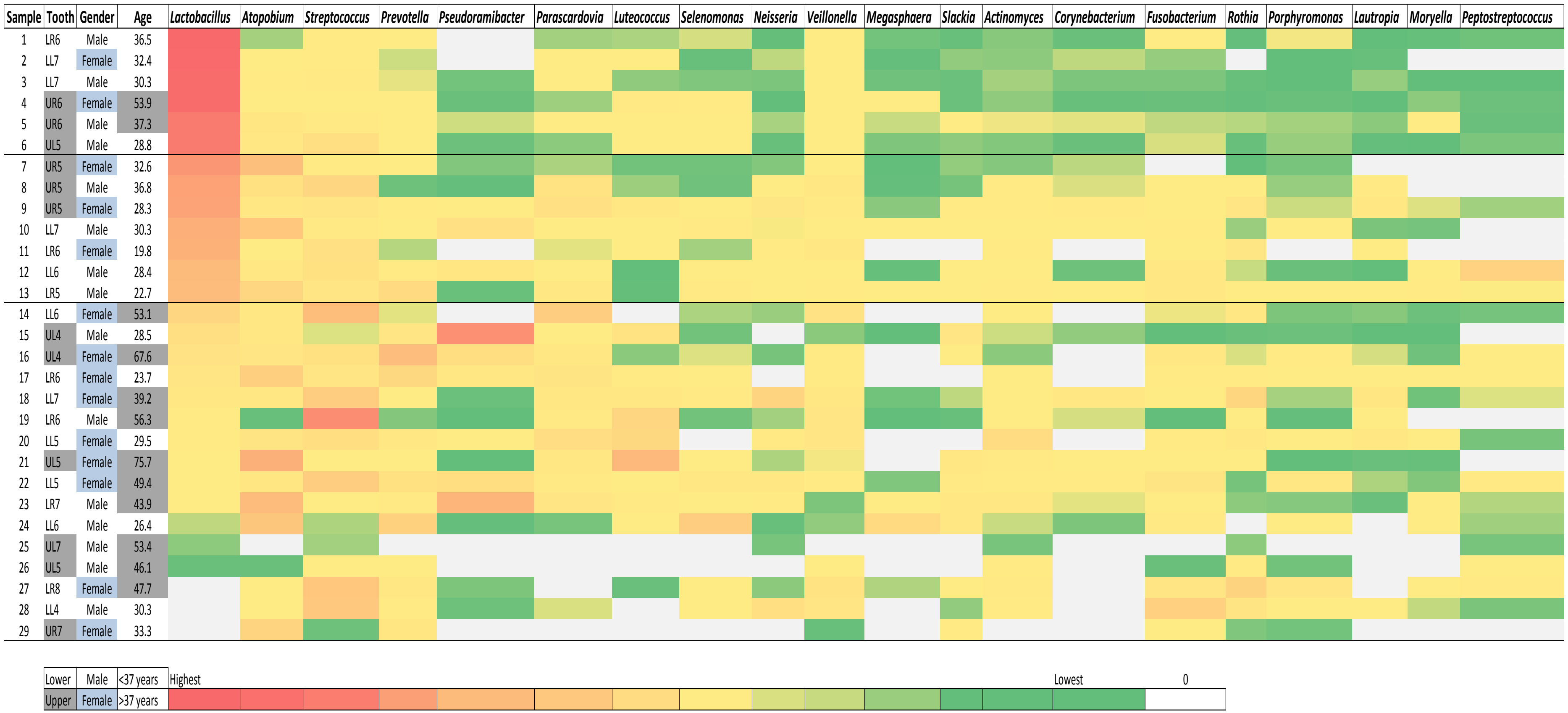


Figure 6-11: Heat-map of top most abundant 20 genera in deep carious dentine samples (29 samples) divided into three clusters according to the relative abundance of *Lactobacillus*. High-*Lactobacillus* group from samples 1-6 (85-97% relative abundance), medium-*Lactobacillus* group from sample 7-13 (35-64% relative abundance) and low-*Lactobacillus* group from sample 14-29 (0.1-17%). Age, gender and tooth type are indicated on the left side of the heat-map.

6.5.3.3 The Difference in Abundance and Prevalence of Bacteria between Superficial and Deep Carious Dentine:

The relative abundance and prevalence of top ten genera in 50 matched samples (25 superficial carious dentine samples and 25 deep carious dentine samples) from the same 25 patients are shown in Figure 6-12 and Figure 6-13. The top ten genera were *Lactobacillus* (34.6% and 31.5% in superficial and deep carious dentine, respectively), *Atopobium* (11.5% and 9.2% in superficial and deep carious dentine, respectively), *Streptococcus* (8.7% and 9.5% in superficial and deep carious dentine, respectively), *Pseudoramibacter, Prevotella, Luteococcus, Parascardovia, Veillonella, Peptostreptococcus* and *Rothia.*

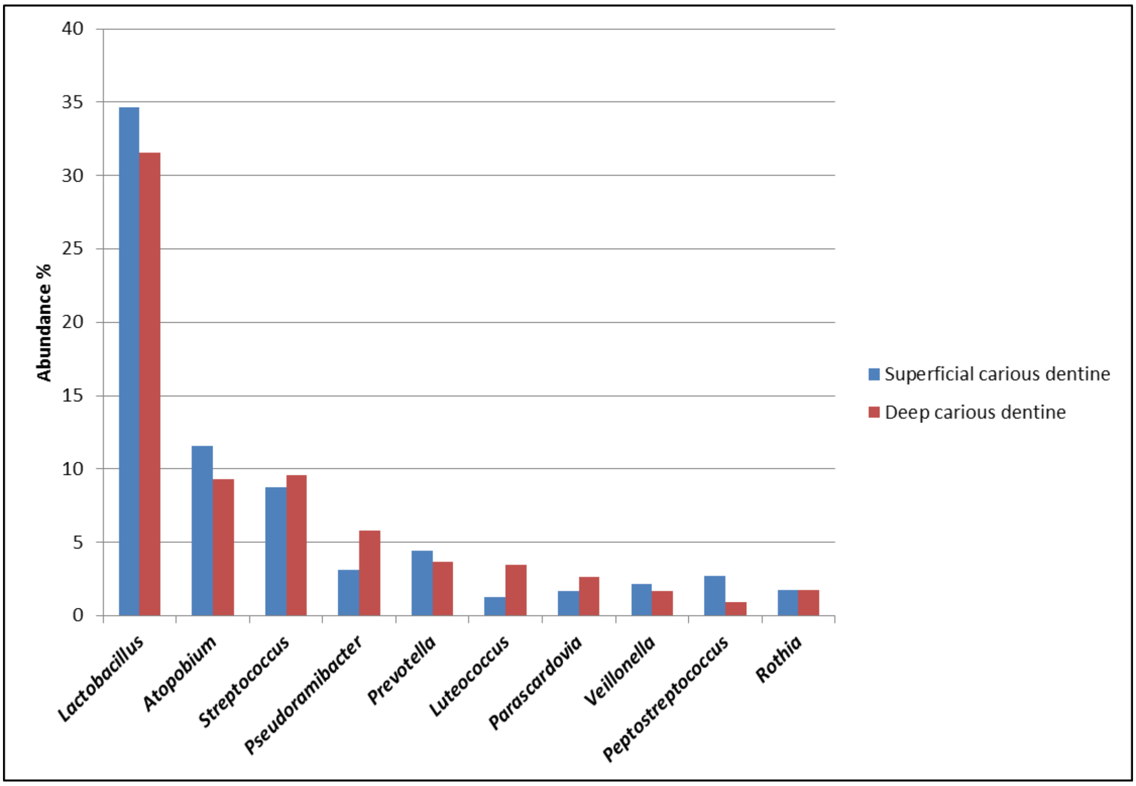


Figure 6-12: Top ten abundant genera in superficial and deep carious dentine of 25 pairs of matched samples from 25 patients.

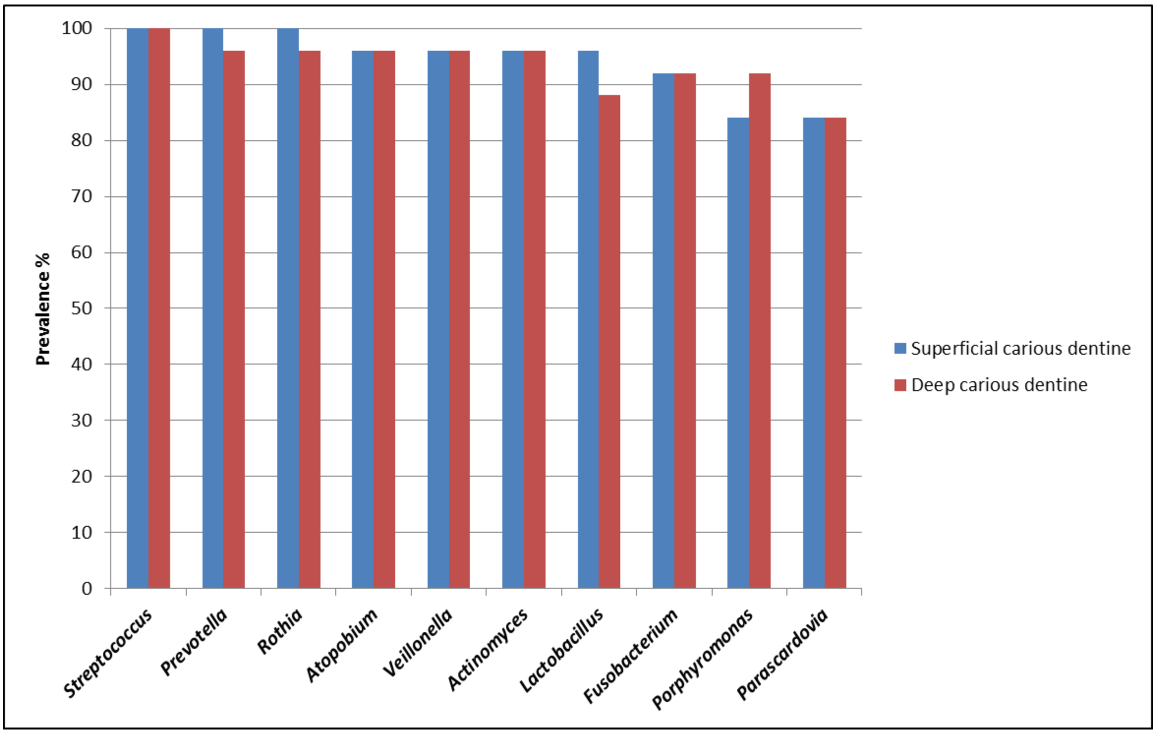


Figure 6-13: Top ten prevalent genera in superficial and deep carious dentine in 25 matched samples from 25 patients.

Wilcoxon signed rank test on data of 25 matched pairs of samples from 25 patients identified the genera that showed a significant difference in abundance between superficial and deep carious dentine in the matched samples from the same teeth to avoid inter-individual heterogeneity. The results indicated that there was no significant difference in relative abundance of any genus between superficial and deep carious dentine in those 25 patients. McNemar test was used to compare the prevalence of each genus in superficial and deep carious dentine in 25 matched pairs of samples. The results showed that there was no significant difference between superficial and deep carious dentine in the prevalence of any genus ($p>0.05$).

6.5.3.4 Gram Status and Oxygen Requirements

In genera that are above 0.01% relative abundance, the higher number of identified bacterial genera belonged to gram-positive anaerobic bacteria (57%). The total percentage of anaerobic was 65% and only approximately 17% of identified bacterial species were aerobic. The percentage of identified facultative species was 18%, as shown in Figure 6-14.

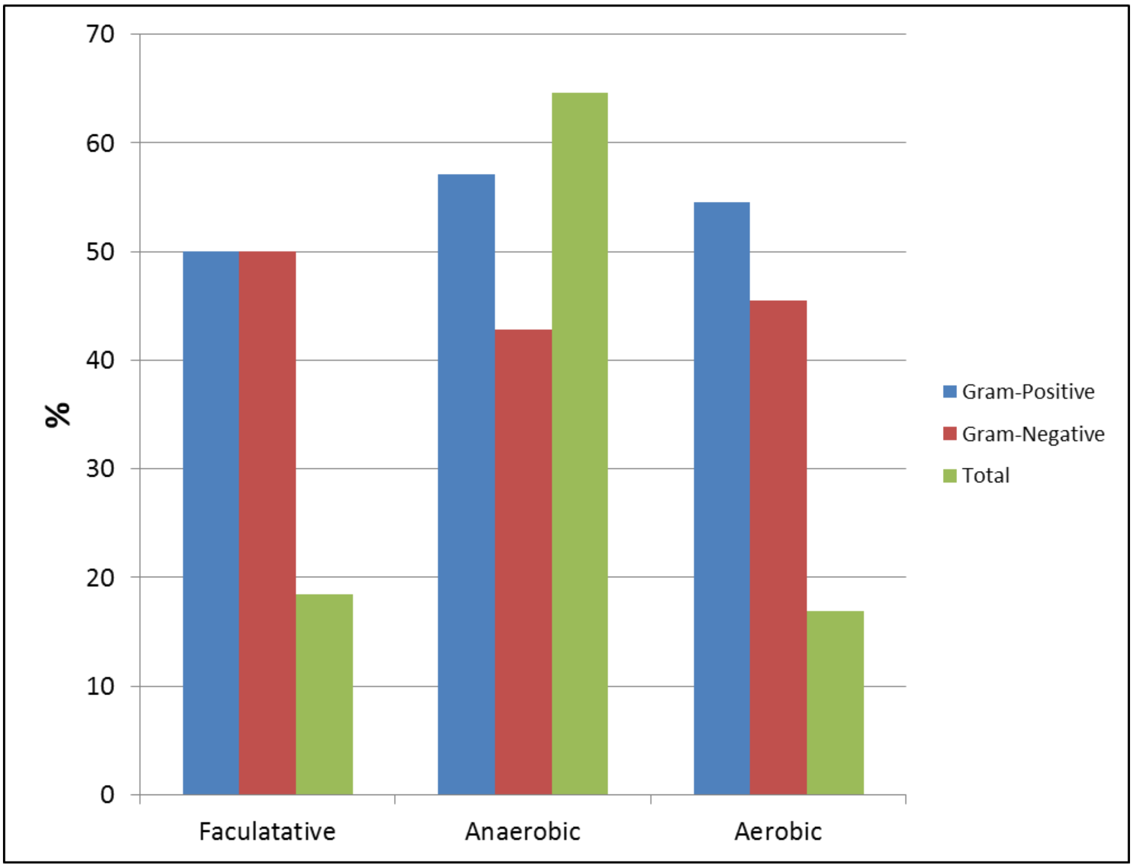


Figure 6-14: Classification of identified bacterial genera (above 0.01% relative abundance) according to gram-staining and oxygen requirement.

6.5.4 Association between Bacterial Prevalence and Clinical Outcome, Symptom Severity, Gender, Age, Arch and Tooth Type of Patients:

Association between bacterial prevalence in superficial and deep carious dentine and clinical signs, symptoms, gender, age, tooth type and outcome of patients was performed in each layer of carious dentine. Clinical outcome and other clinical features of teeth that yielded taxonomic data in next-generation sequencing are illustrated in Figure 6-15 and Figure 6-16.

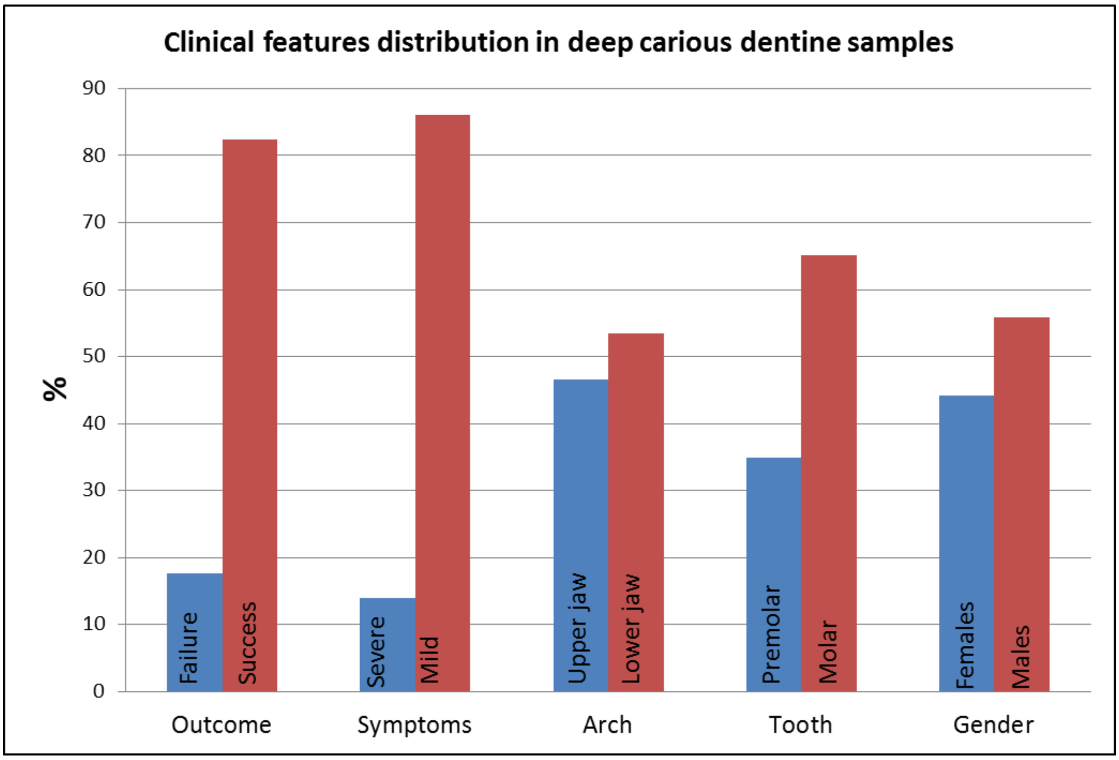


Figure 6-15: Clinical features distribution in 29 samples of deep carious dentine.

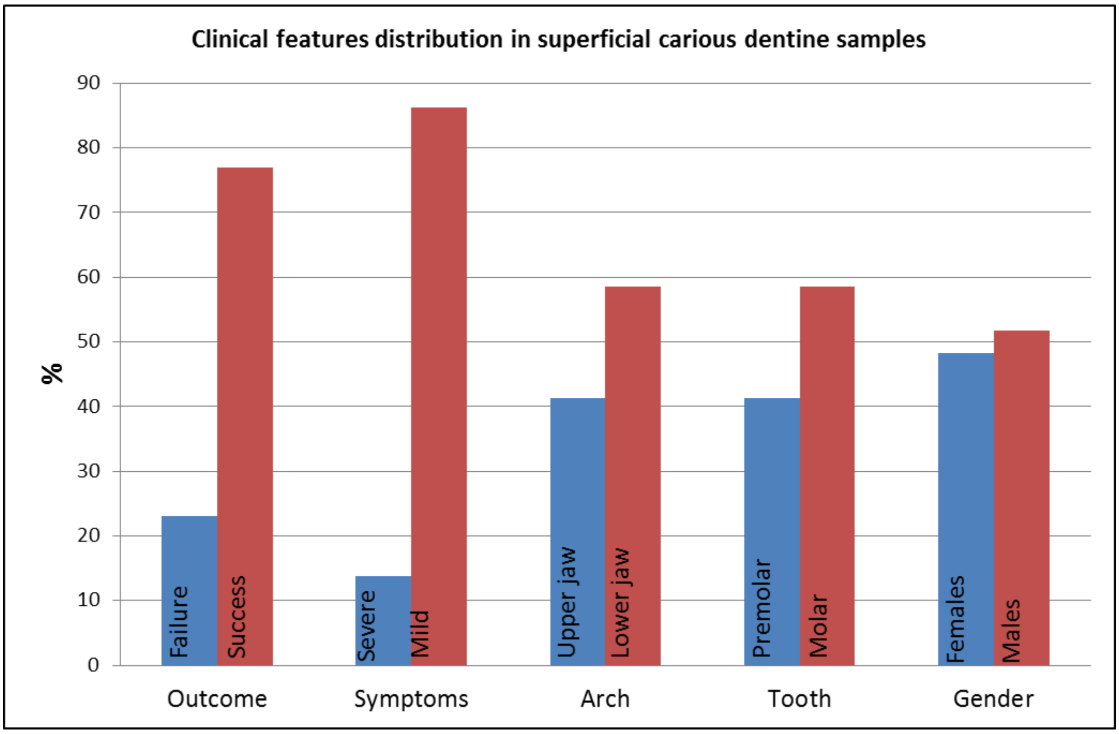


Figure 6-16: Clinical features distribution in 43 samples of superficial carious dentine.

6.5.4.1 Superficial Carious Dentine Samples:

Fisher exact test assessed the association between prevalence of bacteria in superficial carious dentine and treatment outcome, symptoms intensity, tooth position, tooth type and gender. Mann-Whitney U-test was used to assess the correlation between bacterial prevalence in superficial carious dentine and age of the patients.

6.5.4.1.1 **Outcome**

Acholeplasma and *Oxalobacter* were associated significantly with failure outcome compared to success; p-values were 0.018, 0.002, respectively. Their prevalence was 83.3% and 83.3%, respectively in failed cases compared to 25% and 10%, respectively in successful cases.

6.5.4.1.2 **Symptoms:**

There was a significant association between *Granulicatella* presence and symptoms intensity p-value was 0.028. *Granulicatella* was more prevalent in lesions with mild symptoms (100%) compared to lesions with severe symptoms (66.7%). However, *Caloramator*, *Gluconobacter* and *Dermacoccus* were significantly more prevalent in severe cases (100%, 33.3% and 50%, respectively) compared to its prevalence in mild cases (44.4%, 0% and 0%, respectively) with p-value (0.021, 0.028. 0.004).

6.5.4.1.3 **Tooth Position**

Acidaminococcus presence was found significantly associated with upper jaw; p=0.017. It was more prevalent in upper 43.8% compared to in lower 5.9%. *Erysipelothrix, Parapedobacter, Pseudidiomarina, Acinetobacter, Vibrio, Rickettsiella, Sporolactobacillus, Kineosporia* and *Nesterenkonia* were significantly associated with lower jaw (64.7%, 58.8%, 64.7%, 76.5%, 82.4%, 76.5%, 94.1%, 94.1% and 52.9%, respectively) with p-value (0.037, 0.032, 0.037, 0.015, 0.032, 0.015, 0.039, 0.039 and 0.026, respectively) compared to its prevalence in upper jaw (25%, 18.8%, 25%, 31.2%, 43.8%, 31.2%, 62.5%, 62.5% and 12.5%, respectively).

6.5.4.1.4 **Gender**

Pasteurella, *Elizabethkingia*, *Dethiosulfovibrio* and *Alcanivorax* prevalence were significantly associated with gender; p-values were 0.01, 0.032, 0.015, and 0.017, respectively. They were more prevalent in men (87.5%, 81.2%, 75% and 43.8%, respectively) compared to females (41.2%, 41.2%, 29.4% and 5.9%, respectively).

6.5.4.1.5 **Age**

The age of patients with positive detection of *Mogibacterium, Shuttleworthia* and *Luteococcus* was significantly younger than the age of patients with negative detection (p=0.031, 0.002 and 0.005, respectively), as shown in Figure 6-17.

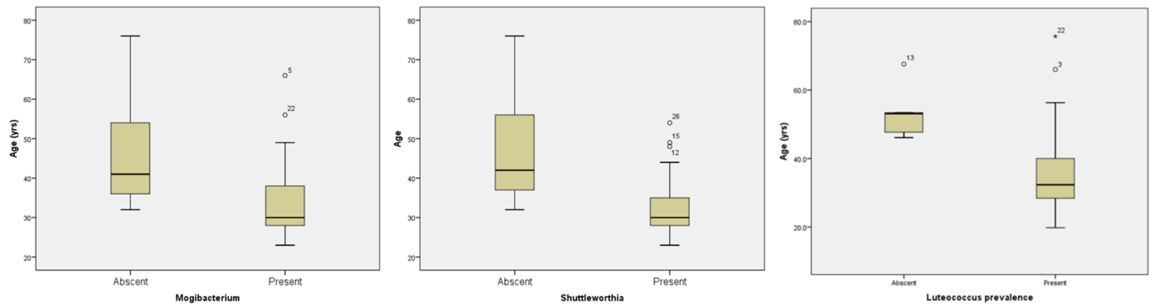


Figure 6-17: Prevalence of significant species in superficial carious dentine by mean age. Patients positive for *Mogibacterium*, *Shuttleworthia* and *Luteococcus* were younger than patients negative for these bacteria.

6.5.4.2 Deep Carious Dentine Samples:

Fisher exact test assessed the association between prevalence of bacteria in deep carious dentine and treatment outcome, symptom intensity, tooth position, tooth type and gender. Mann-Whitney U-test was used to assess the correlation between bacterial prevalence in deep carious dentine and age of the patients.

6.5.4.2.1 **Clinical Outcome:**

There was a significant association between prevalence of some genera in deep carious dentine and clinical outcome after one year of treatment. These bacteria were more prevalent in failed cases compared to its prevalence in successful cases, as shown in Table 6-6.

Table 6-6: Statistically significant genera in deep carious dentine samples according to the clinical outcomes.

Genus name	Prevalence % in failed cases	Prevalence % in successful cases	*p*-value
Paraprevotella	100	9.1	0.011
Stenoxybacter	100	18.2	0.027
Elizabethkingia	100	18.2	0.027
Coriobacterium	100	18.2	0.027
Gallibacterium	100	0	0.003

Actinoplanes	100	9.1	0.011
Uliginosibacterium	66.7	0	0.033
Vogesella	100	9.1	0.011
Tolumonas	100	18.2	0.027
Thiobacillus	100	18.2	0.027
Roseospira	100	18.2	0.027
Deferribacter	100	18.2	0.027
Bartonella	100	18.2	0.027
Ralstonia	66.7	0	0.033
Limnobacter	66.7	0	0.033
Propionicimonas	66.7	0	0.033
Runella	100	0	0.003

6.5.4.2.2 **Symptom intensity:**

There was a significant association between prevalence of *Candidatus Regiella*, *Phenylobacterium*, *Desulfonatronum*, *Dermacoccus* and *Herminiimonas* and symptoms intensity p-values (0.036, 0.018, 0.036, 0.01 and 0.018, respectively). *Candidatus, Regiella* and *Desulfonatronum* were not present in cases with severe symptoms (0% and 0%, respectively) compared to its presence in cases with mild symptoms (75% and 75%, respectively). *Phenylobacterium* was less prevalent in severe cases (30%) compared to mild symptoms cases (100%). However, *Dermacoccus* and *Herminiimonas* were more prevalent in severe cases (65% and 66%, respectively) but not present in mild symptoms cases (0%).

6.5.4.2.3 **Tooth position:**

There was a significant association between the prevalence of *Mogibacterium, Candidatus, Tammella, Thiothrix, Sporotomaculum, Dethiosulfovibrio, Desulfobacter, Sporolactobacillus, Anaerococcus, Lentibacillus, Gluconobacter, Muricauda, Anaeromusa* and *Acidiphilium* and tooth position in the upper or lower jaw *p*-values were 0.006, 0.04, 0.018, 0.018, 0.041, 0.04, 0.01, 0.01, 0.024, 0.04, 0.013, 0.018 and 0.013, respectively. *Mogibacterium, Candidatus, Tammella, Thiothrix, Sporotomaculum, Dethiosulfovibrio, Desulfobacter, Sporolactobacillus, Lentibacillus, Muricauda, Anaeromusa* and *Acidiphilium* were more prevalent in the lesions of teeth of lower jaw (90%, 90.9%, 58%, 100%, 90.9%, 90.9%, 90.9%, 81.8%, 63.6%, 54.5% and 63.6% respectively) compared to its prevalence in teeth of the upper jaw (25%, 37.5%, 0%, 50%, 37.5%, 37.5%, 37.5%, 25%, 0%, 0% and 0%, respectively). However, *Anaerococcus* and *Gluconobacter* were more prevalent in lesions of upper jaw (50% and 62.5%, respectively) compared to lesions in teeth of lower jaw (0% and 9.1%, respectively).

6.5.4.2.4 **Tooth Type:**

There was a significant association between the presence of *Eggerthella, Tessaracoccus, Gluconobactera* and *Actinoplanes* and tooth type; *p*-values were 0.045, 0.041, 0.04 and 0.041, respectively. *Eggerthella, Tessaracoccus, Gluconobacter* and *Actinoplanes* were more prevalent in premolars (100%, 62.5%, 62.5% and 62.5%, respectively) compared to molars (54.5%, 9.1%, 9.1% and 9.1%, respectively). However, *Candidatus, Tammella, Sporotomaculum, Salinicoccus* and *Aerococcus* were more prevalent in molars (90%, 100%, 100% and 45.5%, respectively) compared to premolars (37%, 50%, 37.5% and 0%, respectively); p- values were 0.04, 0.041, 0.005 and 0.045, respectively.

6.5.4.2.5 **Gender:**

Chryseobacterium and *Chlorobaculum* prevalence were significantly associated with males (100% and 75%, respectively) compared to its prevalence in females (57% and 14.3%, respectively) (p=0.03 and 0.02 respectively). However, *Curvibacter*, *Bradyrhizobium, Thiocapsa, Psychromonas* and *Knoellia* were more prevalent in females (42.5%, 42.5%, 71.45, 42.9% and 42.9%, respectively) compared to its prevalence (0%, 0%, 16.7%, 0% and 0%, respectively) in males with (p=0.036, 0.036, 0.045, 0.036, 0.036, respectively).

6.5.4.2.6 **Age**

The age of patients with positive detection to *Leuconostoc* and *Brachybacterium* was significantly younger than the age of patients with negative detection (p=0.046, 0.034, respectively). However, the age of patients with positive detection to *Bradyrhizobium* was significantly older than the age of patients with negative detection (p=0.033), as shown in Figure 6-18.

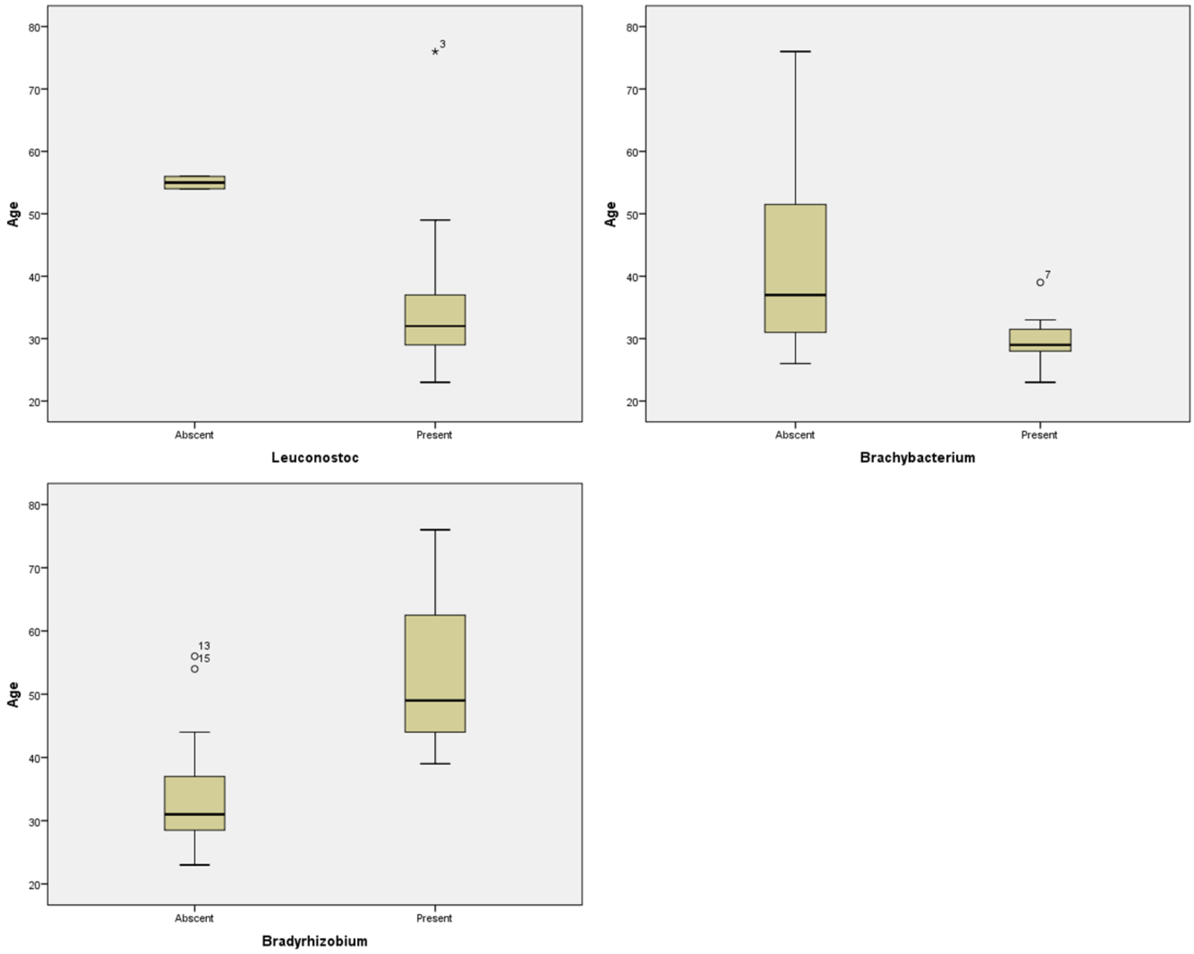


Figure 6-18: Prevalence of significant species in deep carious dentine by mean age. Patients positive for *Leuconostoc* and *Brachybacterium* were younger than patients negative for these bacteria. However, patients positive for *Bradyrhizobium* were older than the patients negative for this species.

6.5.5 Association between Abundance of Bacterial Taxa and Clinical Features

6.5.5.1 Clinical Outcome (Success / Failure)

The PCA and heat-map analyses of 72 superficial and deep carious dentine samples from successful and failed teeth (48 successful, nine failed and 11 samples from teeth lost to follow-up and four samples from teeth with pulp exposed) showed that there was a clustering of groups of samples from successful teeth, which indicate that these samples have a common microbiological feature among them that can drive the clinical outcome either to success or failure, as shown in Figure 6-19. It is noted that some samples from failed and pulp-exposed teeth aggregate in clusters characterised by either a high proportion of *Prevotella* or *Lactobacillus*, as shown in Figure 6-20.

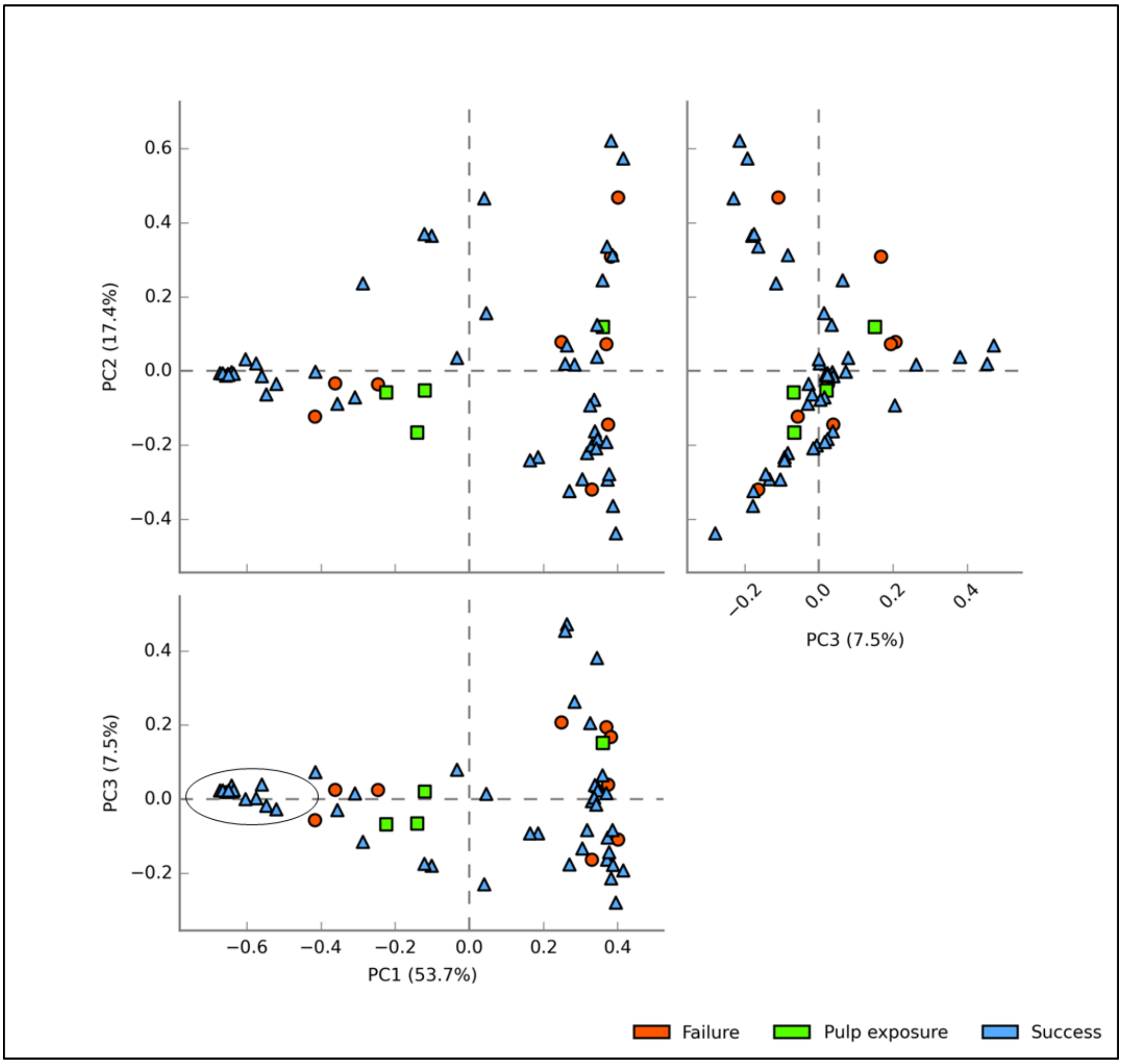


Figure 6-19: PCA analysis of superficial and deep carious dentine samples from successful, failed and pulp-exposed teeth after one year of treatment. Although inter-individual variability can be observed, some successful samples tend to group together (circles) according to the clinical outcome.

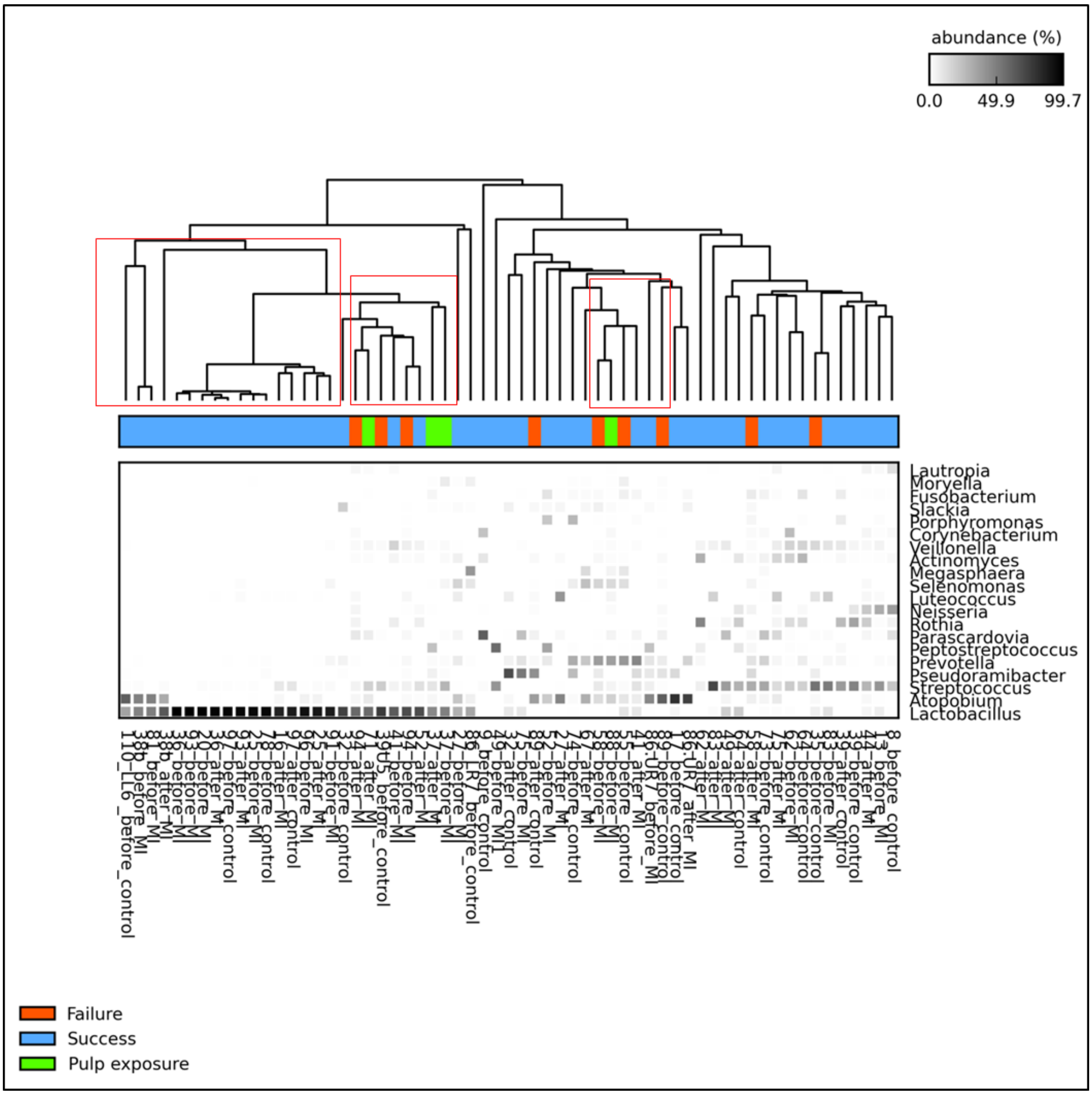


Figure 6-20: Heat-map of superficial and deep carious dentine samples from successful, failed and pulp-exposed teeth shows that the failed and pulp-exposed teeth tend to cluster together in groups. Identified groups encircled with a red rectangle. In one group of the failed and pulp-exposed teeth, there are higher proportions of *Prevotella*. The other two groups are characterised by higher proportions of *Lactobacillus*.

The abundance of each of the top 20 bacterial taxa from superficial and deep carious dentine are pooled together and estimated in the successful and failed lesions. It was found that *Streptococcus*, *Prevotella*, *Pseudoramibacter*, *Selenomomas*, *Veillonella* and *Fusobacterium* have a higher pooled average abundance in the failed cases compared to the successful cases, as shown in

Figure 6-21.

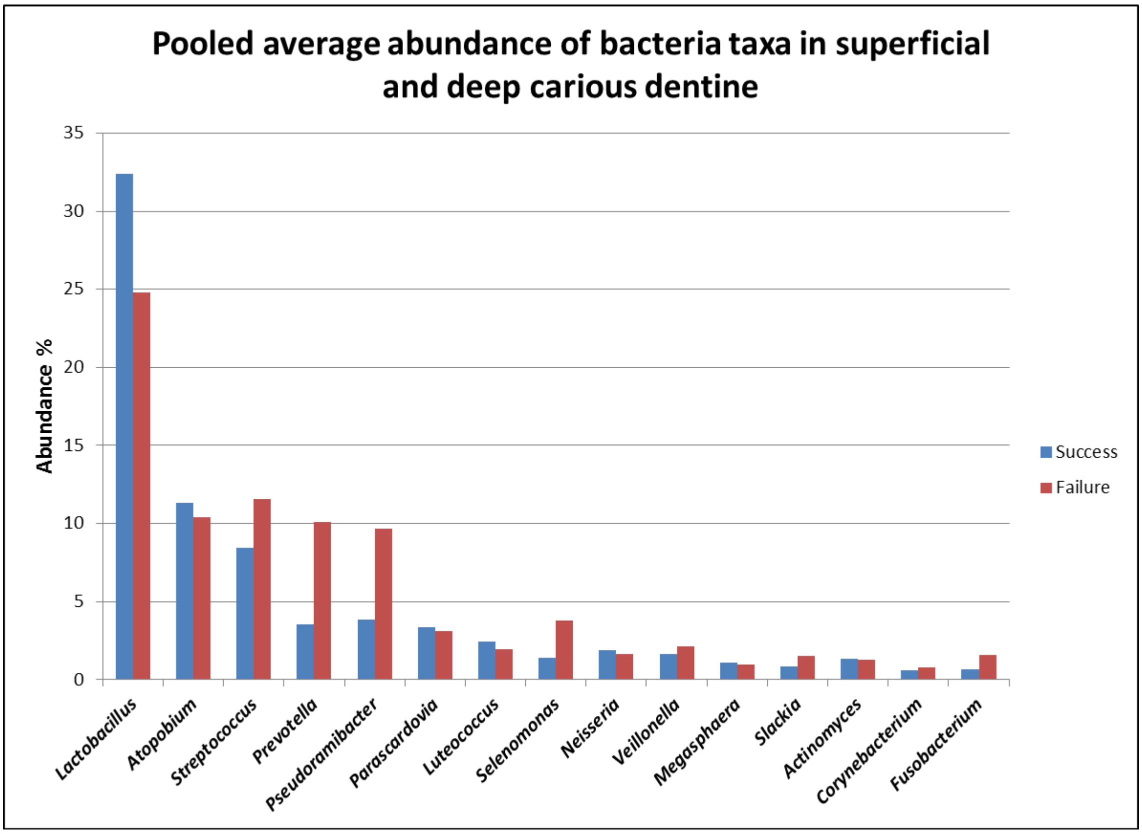


Figure 6-21: Pooled average relative abundance of the top abundant genera from superficial and deep carious dentine samples in failed and successful teeth.

In addition, PCA and heat-map analysis of success / failure of deep carious dentine samples (29 samples categorised as follows: 19 samples from successful teeth, three samples from failed teeth and seven samples from lost to follow-up teeth) showed a similar pattern to that observed in PCA analysis of total samples (together superficial and deep carious dentine shown in Figure 6-19). The deep carious dentine samples from successful cases tended to aggregate together in groups, as shown in Figure 6-22 and Figure 6-23.

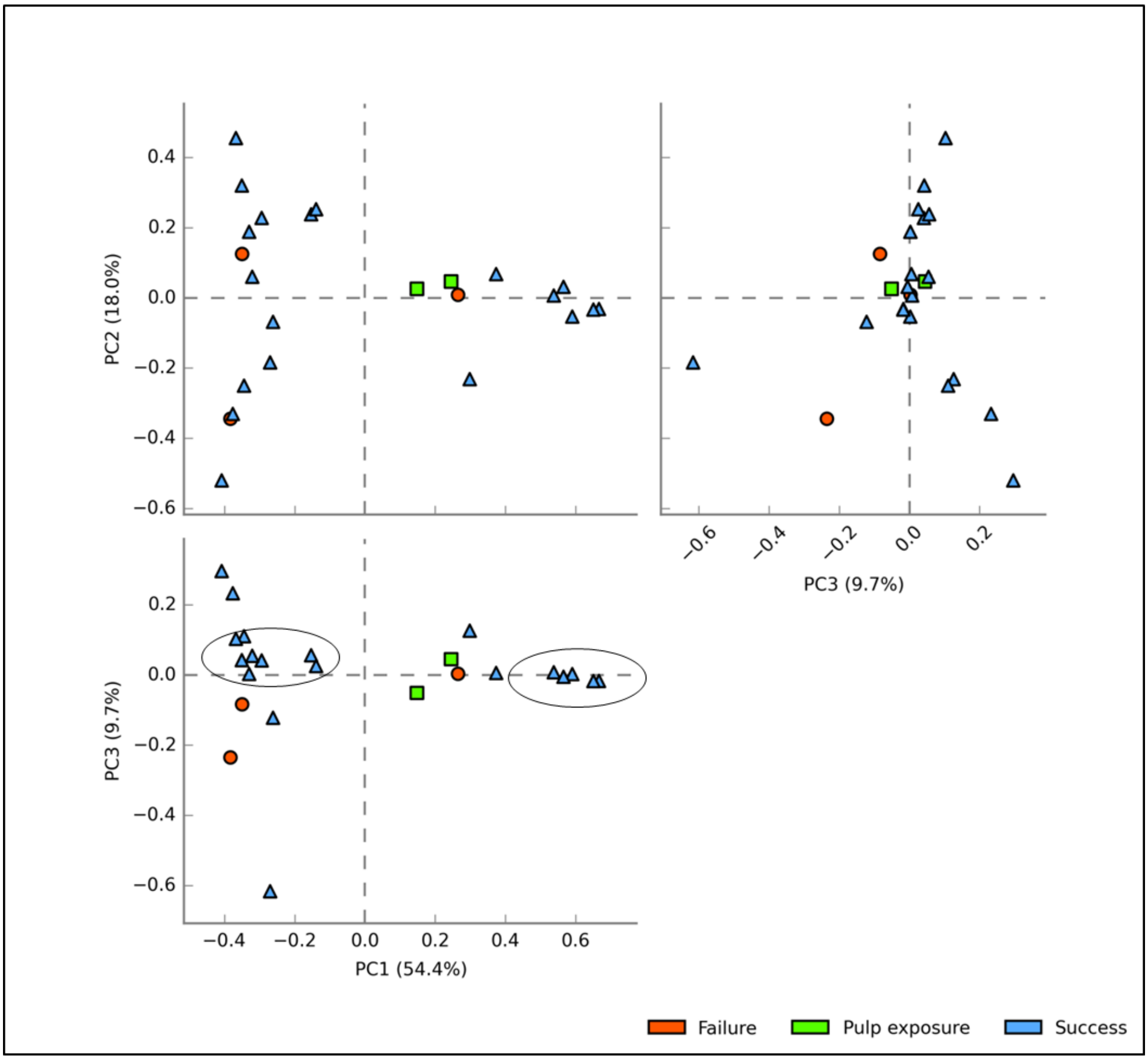


Figure 6-22: PCA analysis of deep carious dentine samples from 19 successful and three failed teeth showing groups of successful teeth (circles).

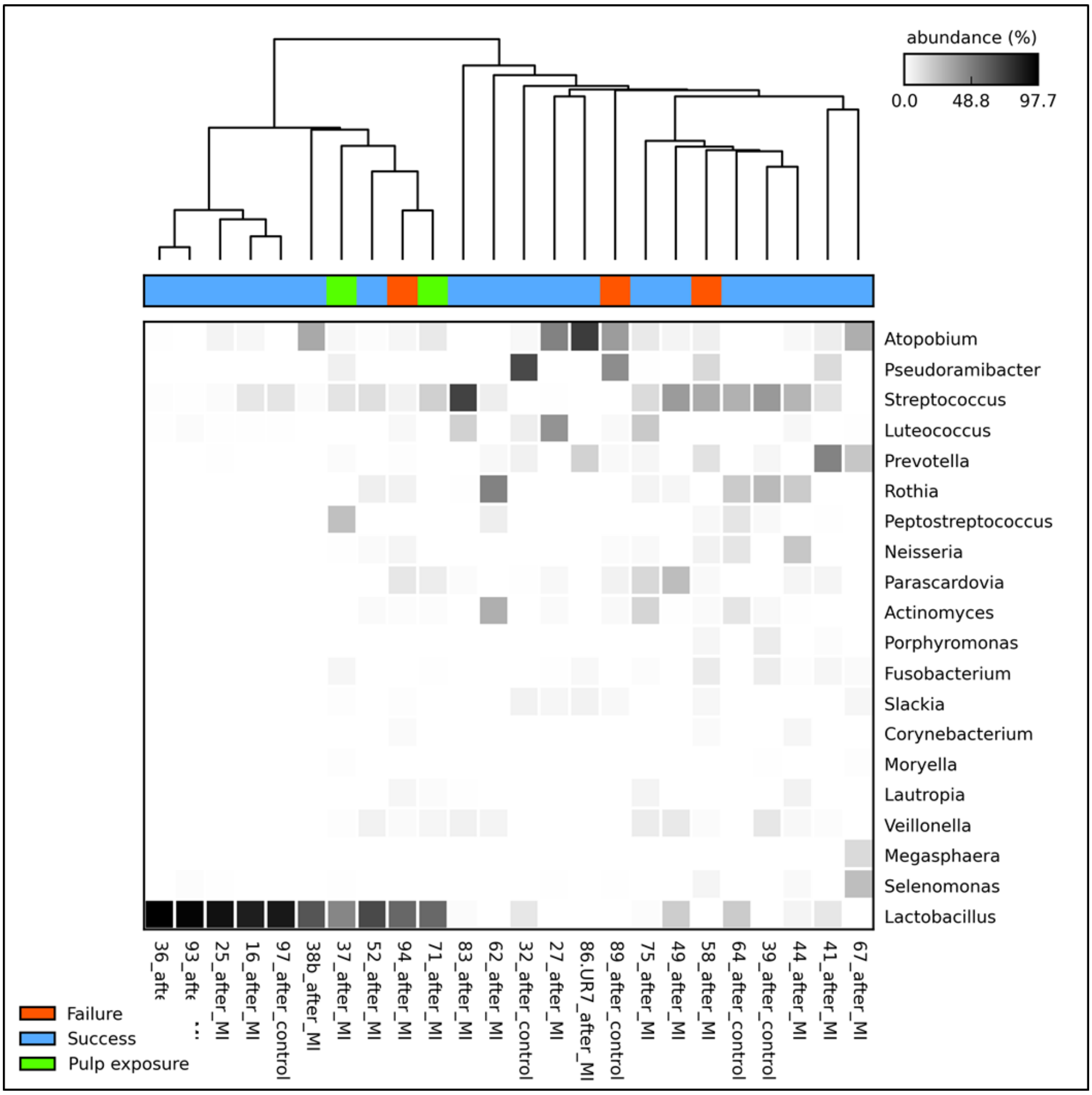


Figure 6-23: Heat-map of deep carious dentine samples from successful, failed and pulp-exposed teeth.

In deep carious samples, *Atopobium*, *Pseudoramibacter*, *Parascardovia*, *Neisseria, Actinomyces, Corynebacterium, Fusobacterium, Porphyromonas* and *Peptostreptococcus* showed higher average relative abundance in samples from failed cases compared to their average relative abundance in successful cases, as shown in Figure 6-24.

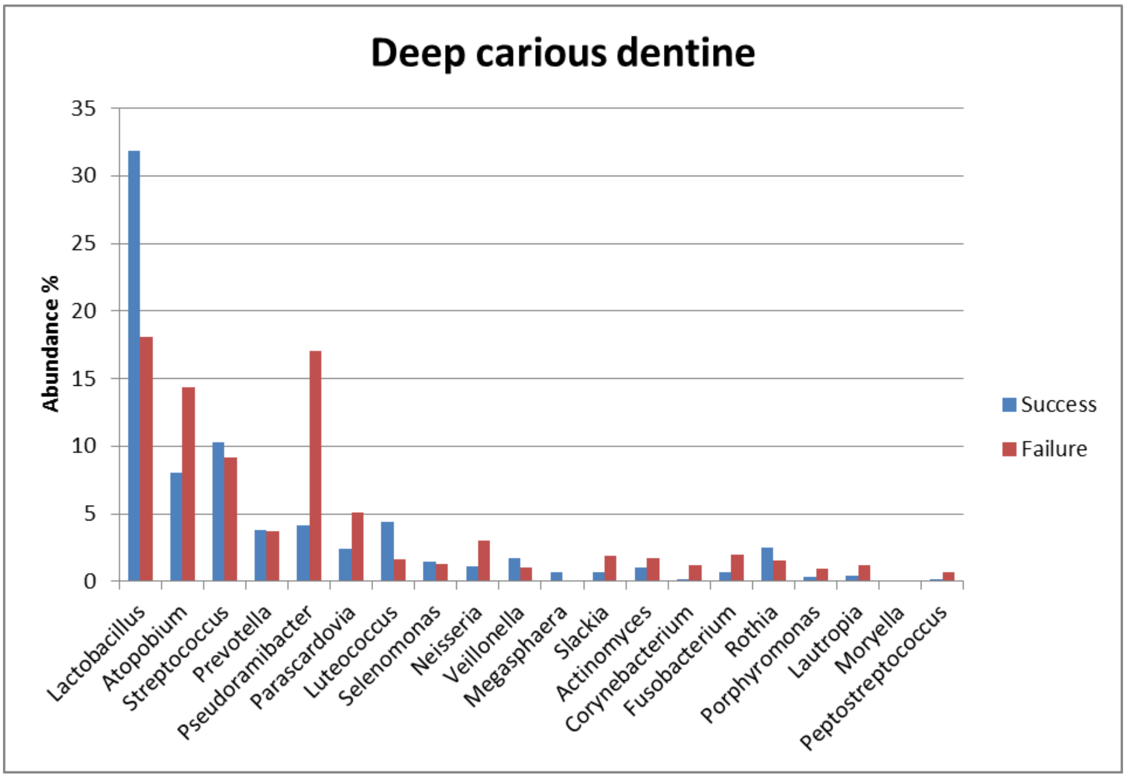


Figure 6-24: The average relative abundance of bacterial genera in deep carious dentine samples from 19 successful and three failed teeth, respectively.

6.5.5.2 Excavation Technique

PCA and heat-map analysis of deep carious dentine samples showed that some samples from teeth excavated with minimally invasive approach clustered together compared to samples from teeth excavated with the control group, as shown in Figure 6-25 and Figure 6-26.

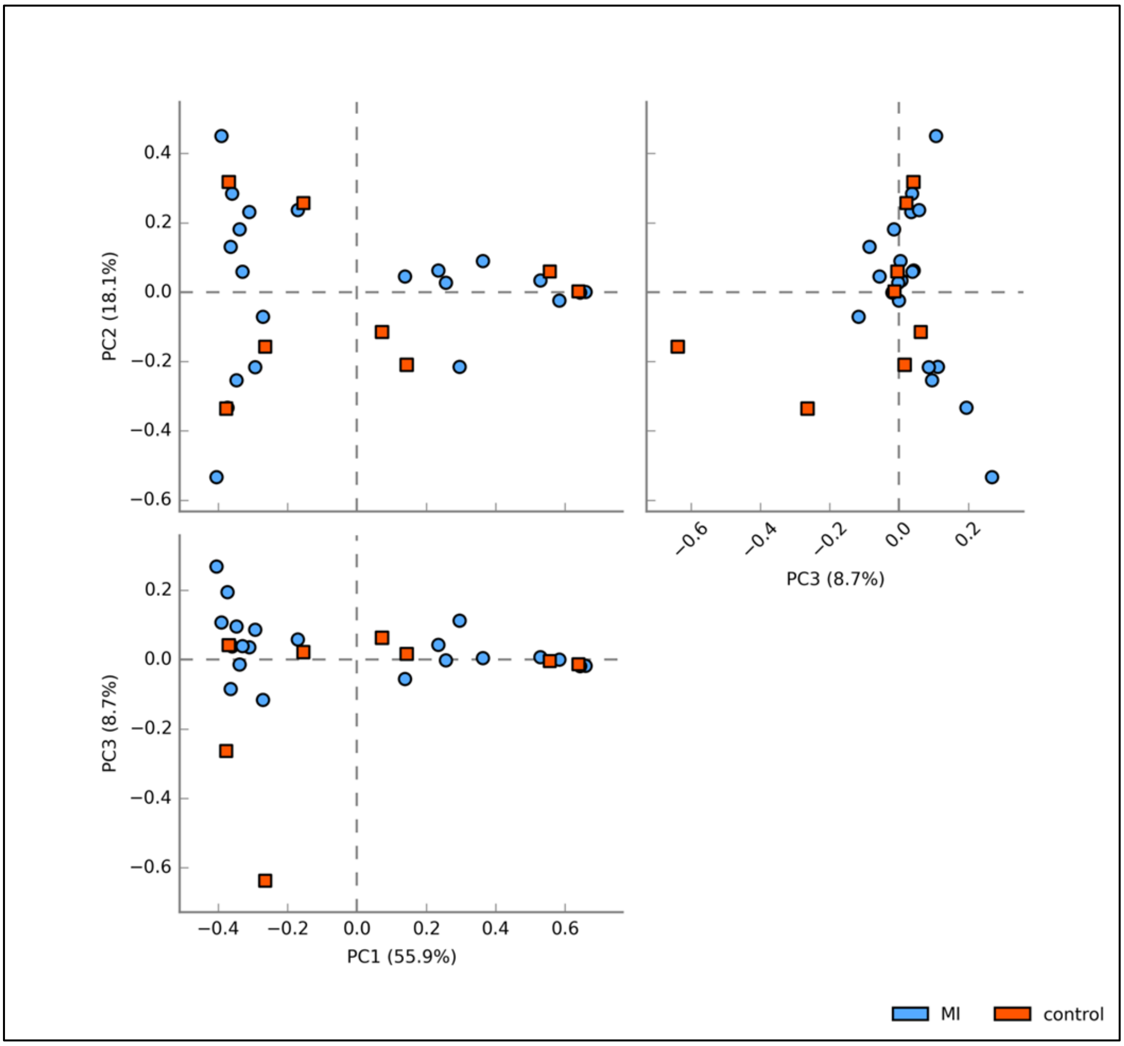


Figure 6-25: PCA analysis of deep carious dentine samples from 21 and eight teeth excavated with minimally invasive and control protocol, respectively.

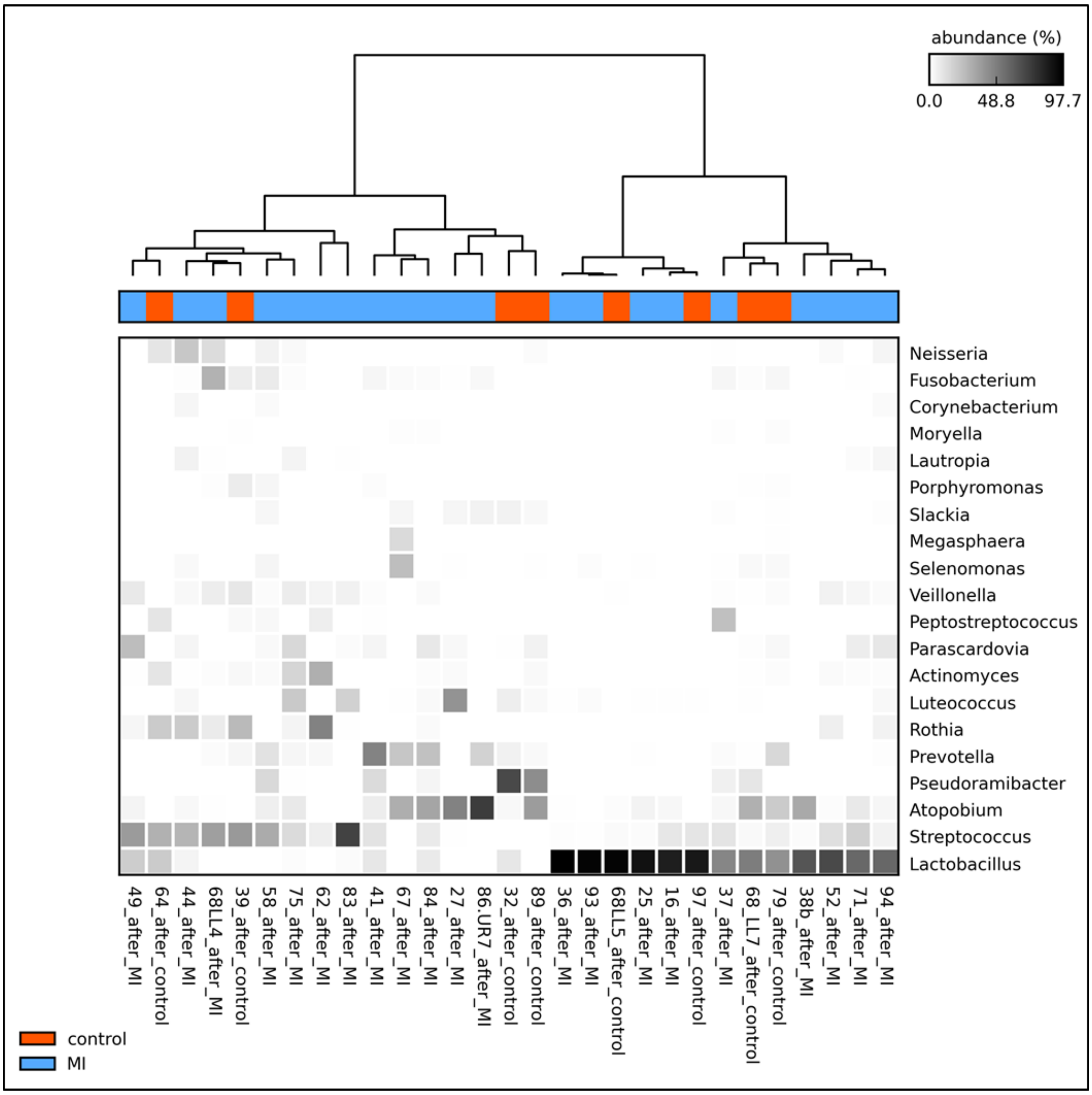


Figure 6-26: Heat-map of deep carious dentine samples from 21 and eight teeth excavated with minimally invasive and control protocols, respectively.

In the minimally invasive group, all top 20 abundant bacteria except *Lactobacillus, Pseudoramibacter*, *Slackia*, *Actinomyces*, *Rothia*, *Porphyromonas* and *Moryella* showed higher average relative abundance in samples from teeth excavated compared to their average relative abundance in samples from the control group, as shown in Figure 6-27.

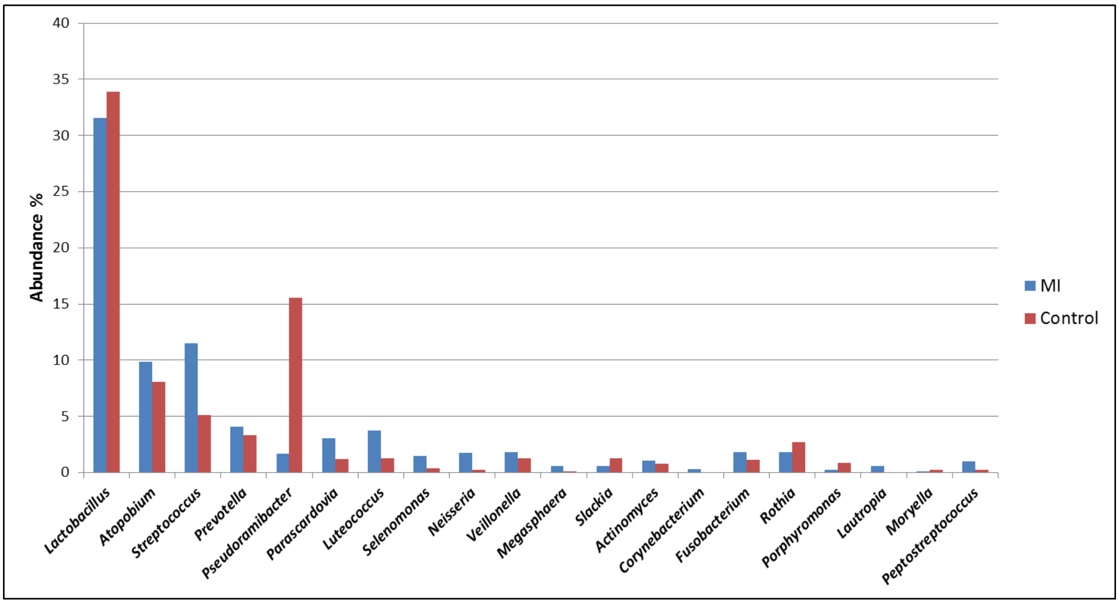


Figure 6-27: The average relative abundance of bacterial taxa in deep carious dentine samples from 21 and eight teeth in minimally invasive and control groups, respectively.

6.5.5.3 Symptoms Severity

PCA and heat-map analysis of deep carious dentine samples showed that groups of samples from teeth presented with mild symptoms cluster together, as shown in Figure 6-28 and Figure 6-29.

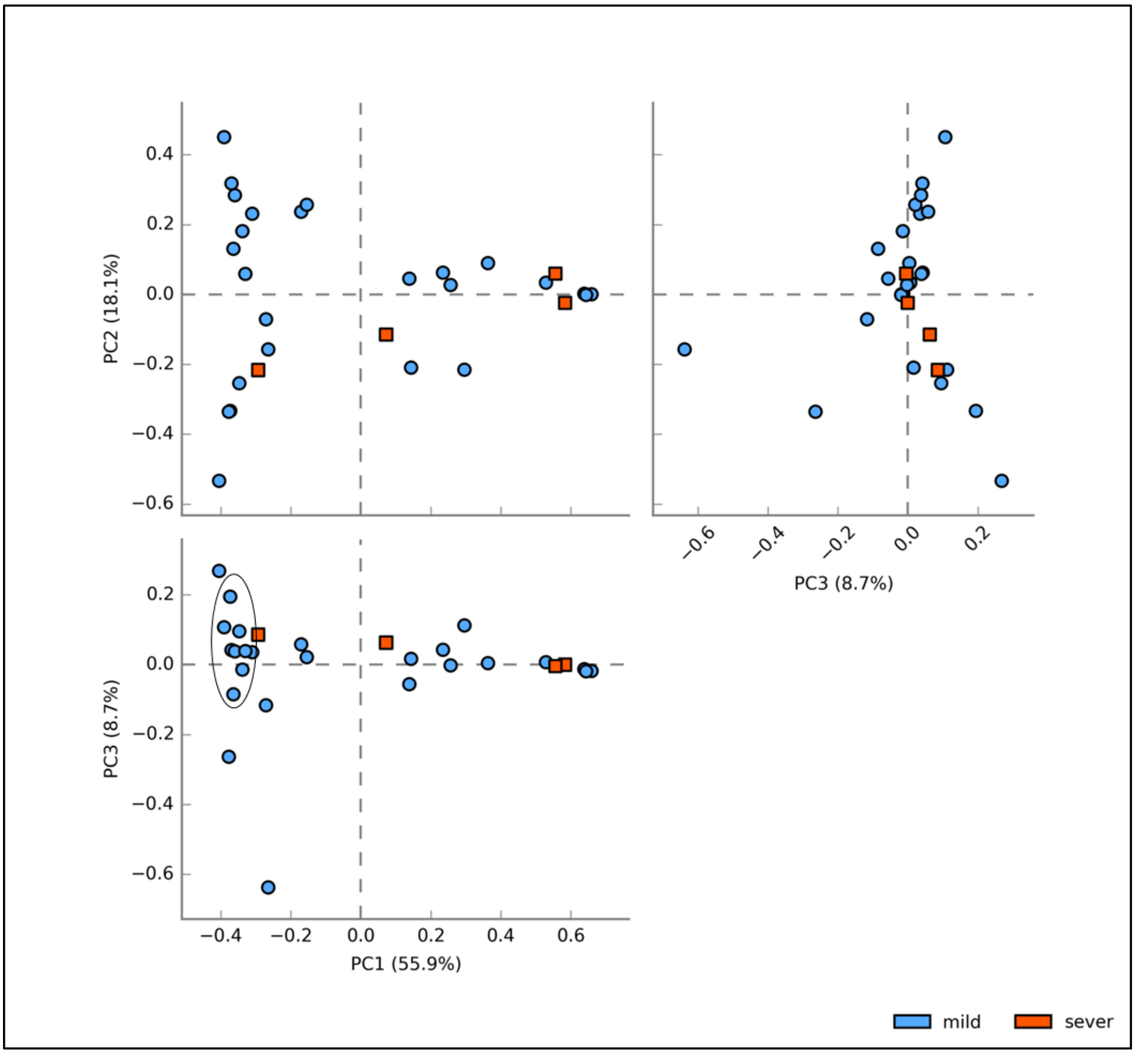


Figure 6-28: PCA analysis of deep carious dentine samples from 25 and four teeth presented with mild and severe symptoms, respectively.

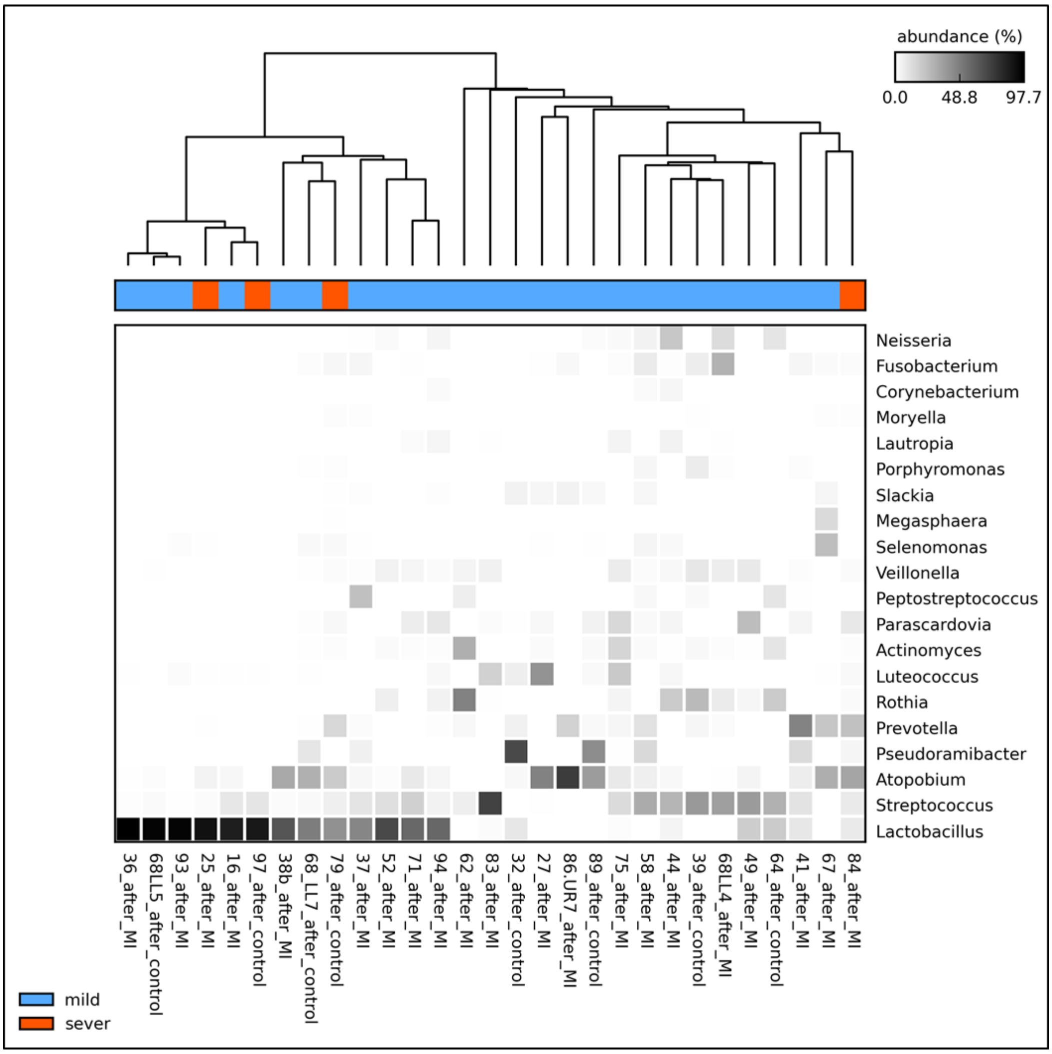


Figure 6-29: Heat-map of deep carious dentine samples from 25 and four teeth excavated with mild and severe symptoms, respectively.

Most bacterial genera in deep carious dentine samples showed no difference in average relative abundance in mild and severe cases, except for *Lactobacillus*, *Streptococcus* and *Pseudoramibacter*, as shown in Figure 6-30. The *Lactobacillus* was more abundant in cases with severe symptoms compared with mild cases. *Streptococcus* and *Pseudoramibacter* were more abundant in mild cases compared with severe cases.

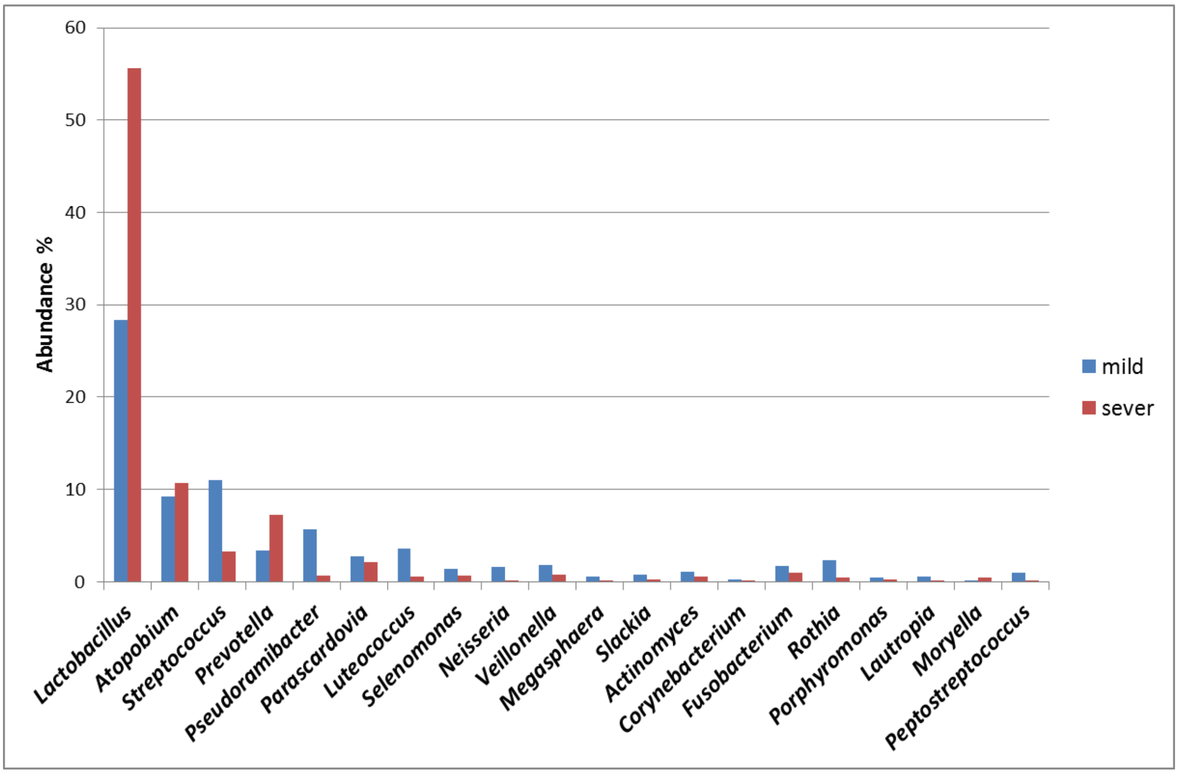


Figure 6-30: The average relative abundance of bacterial genera in deep carious dentine samples from 25 and four teeth presented with mild and severe symptoms, respectively.

6.6 Discussion:

6.6.1 Bacterial Enumeration in Dentine Caries

In this study, the bacterial load and composition of superficial and deep dentine caries were determined before/after caries excavation. The methods used to determine the bacterial load were qPCR, using universal primers to detect the number of the bacterial gene 16S rRNA in each sample, and next-generation sequencing (NGS) was used to determine the bacterial composition of each sample.

Results of the bacterial load enumeration in superficial and deep dentine caries samples using 16S rRNA - qPCR in this study (mean $3.65\times 10^{+06}$ mg^{-1} in superficial carious dentine and $4.03\times 10^{+05}$ mg^{-1} in deep carious dentine) are comparable to those results acquired in another study which used a fluorescent *in situ* hybridisation method for quantification of total bacteria in different depth levels of carious dentine, by targeting the 16S rRNA gene which is specific for bacterial domain, which similarly found higher bacterial counts in the superficial part of the dentinal carious lesion ($7.34\times 10^{+06}$ mg^{-1}) compared to the deep front of the lesion ($1.69\times 10^{+06}$ mg^{-1}). In addition, they found considerable bacterial counts beyond the clinical excavation end point in cavity preparation ($3.4\times 10^{+05}$ mg^{-1}) (Banerjee *et al.* 2002). Other studies that have used culture-based methods for bacterial quantification and characterisation in dentine caries found fewer bacterial numbers in samples of carious dentine (Leung *et al.* 1980, van Strijp *et al.* 1994) in comparison to the numbers found in this study. Others found higher bacterial loads ($1.4\text{x}10^8$ to $1.1\text{x}10^9$ mg^{-1}) in carious dentine (Chhour *et al.* 2005) compared to the average microbial total load in this study. The variability of the results may be due to different methods of sampling in different studies and/or the site of sampling. It has been found that there is high inter-individual variability in bacterial composition among subjects in different studies (Munson *et al.* 2004, Obata *et al.* 2014, Schulze-Schweifing *et al.* 2014).

There were some cavities with deep dentine caries which harboured higher bacterial loads after caries excavation, regardless of the method of the excavation used in this study. The explanation for this can be attributed to the possibility of the presence of yeast in high abundance compared to bacteria in the superficial layer of carious lesion, which cannot be detected using the universal primer specific for bacterial 16S rRNA gene. It was reported before that prolonged status of low pH in advanced cavities will initiate aciduric stage of microflora development in dentine caries, in which there will be an acid-induced selection of microflora growth, leading to the dominance of yeast in some case (Takahashi and Nyvad 2011). Also, the low colonisation of bacteria in the superficial layer compared to the deeper layer in these cavities could be attributed to the effect of anaerobic gram-negative bacteria in the deeper layers that can kill the bacteria in the superficial layer by acid production or antibiotics release. It was suggested that gram-negative bacteria can produce membrane vesicles which are capable of killing other bacteria (Li *et al.* 1998).

However, the majority of cavities showed a reduction of bacterial load after excavation in the deeper layer of carious dentine. Although it was expected to have less reduction in bacterial load in the minimally invasive group compared to control, the minimally invasive protocol was able to reduce the number of bacteria, as well as the conventional drilling technique for caries excavation, as there was no significant difference between them in reducing overall microbial loads. It is worth noting that dental bur may be expected to remove more carious dentine and thus more bacteria. However, Carisolv^{TM} gel could be expected to kill more bacteria because of its antimicrobial properties. In comparison to previous studies that used culture techniques, a molecular method was used for bacterial enumeration in this study which can detect non-viable bacteria resulted from Carisolv^{TM} gel antimicrobial effect. These results agree with previous reports which found equal efficiency of both techniques in reducing bacterial loads (Lager *et al.* 2003, Azrak *et al.* 2004, Sterer *et al.* 2008, Ammari *et al.* 2014). In contrast to another study which found a lower efficiency of Carisolv^{TM} compared to rotary burs in removing bacteria from cavities of teeth, it has been postulated that pushing of bacteria into dentine tubules during excavation with Carisolv^{TM} may lead to the detection of higher numbers of bacteria after excavation (Yazici *et al.* 2003). However, the variability of findings may be attributed to different sampling procedures, excavation extent and microbiological techniques used.

Also, from the clinical point of view, it is not unacceptable to leave bacteria behind in the cavity, especially in the favour of tooth destruction. Microbiological studies on carious cavities found that persistence of bacteria after cavity preparation does not seem to be a reason to re-open the cavities in a later stage in partial caries removal (Orhan *et al.* 2008, Lula *et al.* 2009). This concurs with the results of this study because all teeth that harboured high bacterial loads after deep caries excavation showed a successful clinical and radiological outcome after one year of treatment, except one tooth which suffered pulp exposure during the excavation process itself.

It was expected that chloramines present in Carisolv™ gel, which have an inherent antimicrobial effect, may enhance the antibacterial effect of deep dentine caries. However, this was difficult to detect in this study, which identified the DNA of viable and non-viable bacteria together. It is unlikely that the sodium hypochlorite in deep dentine after Carisolv™ gel excavation may affect the bacteria in the dentine tubules without mechanical agitation. It was found that both higher concentrations (5·5% or 12%) and mechanical agitation are necessary to eradicate bacteria from root dentine (Huque *et al.* 1998). Similarly, it was found that only after a combination of ultrasonic treatment and Carisolv™ gel, the treatment was effective in cleaning root canals (Al-Kilani *et al.* 2003). In coronal dentine, recently it was found that passive application of Carisolv™ gel on infected dentine discs with *Streptococcus mutans* without agitation is less effective in killing bacteria compared to 5.25% NaOCl. Limited penetration of Carisolv™ gel because of viscosity and/or presence of amino acids in Carisolv™ which limit NaOCl reactivity may cause limited antimicrobial activity compared to pure NaOCl (Hamama *et al.* 2014c).

6.6.2 Phylogenetic Profile of Microbiome in Dentine Caries:

This study was the first study that used next-generation sequencing for investigation of advanced carious lesions in teeth presented with signs and symptoms of reversible pulpitis excavated with two different excavation approaches and followed for one year, compared to previous studies that used culture-dependent methods, clonal molecular analysis and pyrosequencing. This study revealed much larger bacterial diversity and complexity in deep dentine caries than previous studies and showed agreement with recent next-generation molecular studies of deep carious lesions (Obata *et al.* 2014, Rôças *et al.* 2016).

Phylogenetic data show that the highest number of identified bacterial genera in dentine caries consisted mainly of gram-positive and negative anaerobic bacteria compared to fewer numbers of facultative and aerobic bacteria. This confirms that deep dentine caries promotes an anaerobic environment that is suitable for growth of these species. These results agree with previous reports (Hoshino 1985, Massey *et al.* 1993, Chhour *et al.* 2005); however, they disagree with other studies, which noted fewer or no gram-negative anaerobic bacteria in dentine caries (Hahn *et al.* 1991, Martin *et al.* 2002). Instead, they found that facultative bacteria were more dominant in deep carious lesions. In the earliest of these studies, this may have been due to difficulties in the isolation of anaerobes or difference in ethnicity and inclusion criteria of studies' populations or because of differences in sampling methodology.

The top most abundant phyla were *Firmicutes, Actinobacteria, Bacteriodetes, Proteobacteria, Fusobacteria* and *Spirochaetes,* and these are similar to that reported in previous reports of deep dentine caries microbiological investigations (Munson *et al.* 2004, Obata *et al.* 2014, Kianoush *et al.* 2014). There was no significant difference in abundance and prevalence of each phylum between the superficial and deep carious dentine. The most abundant phyla were *Firmicutes,* with about 80% and 81% relative abundance in superficial and deep carious dentine, respectively, which agrees with the findings of studies which showed the dominance of *Firmicutes* in dentine caries (Gross *et al.* 2010, Obata *et al.* 2014, Kianoush *et al.* 2014). Other minor phyla such as *Synergistetes, Cyanobacteria, Tenericutes* and *Thermotogae* were also present in much lower proportions (<0.5% relative abundance) in this study, similar to the proportion of minor phyla such as *TM7, Cyanobacteria* and *Deinococcus-Thermus* presented in previous microbiological investigations of dentine caries (Gross *et al.* 2010, Obata *et al.* 2014). Similarly, there was no significant difference in prevalence of phyla between superficial and deep carious dentine. However, another study found that *Firmicutes* was present in a lower proportion (36-53%) in high acidic zones of carious lesion, compared to 78% in less acidic zones (Kianoush et al. 2014). Our findings show that there was no difference in *Firmicutes* abundance between superficial and deep layers, which were expected to have different levels of acidity.

The total number of detected genera of dentine caries in this study was 598. However, the number of genera that have relative abundance above 0.01% was 94, which is comparable to that found in another studies (Chhour *et al.* 2005, Munson *et al.* 2004, Obata *et al.* 2014) which identified 75, 95 and 79 taxa in samples of deep carious dentine, respectively. However, in this study, higher numbers of bacterial genera (above 0.01% relative abundance) were found per sample (81.5 ± 7.9) compared to previous studies, which found around 31 and 32.2 taxa in deep carious dentine, respectively (Chhour *et al.* 2005, Munson *et al.* 2004). This is attributed to the fact that these studies used cloning techniques for microbiological investigation of 16S rRNA genes in the samples of dentine caries, in contrast to the next-generation sequencing used in this study, which can yield a wider spectrum of the bacterial composition.

The result of this study found that the most abundant genus in superficial and deep carious dentine was *Lactobacillus*, followed by *Atopobium* and *Streptococcus*. However, *Lactobacillus* was not present in all samples. *Streptococcus* was present in all the samples of the superficial and deep dentine caries in all patients. Other studies found a similar prevalence of *Streptococcus* in dentine caries samples (Munson *et al.* 2004, Schulze-Schweifing *et al.* 2014). Other studies found that *Lactobacillus* is the most abundant genus in deep dentine caries rather than *Streptococcus* and it was not present in all samples tested (Gross *et al.* 2010, Obata *et al.* 2014), which is similar to the findings of this study. Some studies found undetectable levels of *Streptococcus mutans* in 10-15% of caries-active subjects, which suggest that the presence of *Streptococcus mutans* does not necessarily indicate caries activity (Beighton 2005, Aas *et al.* 2008).

Byun et al. (2004) found that members of the *Lactobacillus* genus were present in 100% of their samples using the qPCR technique, with higher mean loads of *Lactobacillus gasseri* and *Lactobacillus ultunensis* than of the other species. Also, Schulze-Schweifing *et al.* (2014) found that *Lactobacillus gasseri* was the most abundant species in dentine caries using clonal analysis. Similarly, findings of the present study using next-generation sequencing found that *Lactobacillus* was the most abundant genus in superficial and deep dentine caries. However, there was no statistical significance in its abundance and prevalence between superficial and deep carious dentine. These findings suggest that colonisation and proliferation of this genus in carious dentine is promoted by the anaerobic environmental conditions associated with deep carious lesions. Also, it was reported that *Lactobacillus* species have high affinity to collagen type I and they have a higher proportion in severely demineralised dentine (Van Strijp *et al.* 1997, McGrady *et al.* 1995). Munson *et al.* (2004) found that the relative abundance of *Streptococcus* was lower than *Lactobacillus* in the middle and advanced fronts of deep carious lesions microflora, in agreement with the results of this study, which found that the relative abundance of *Lactobacillus* and *Streptococcus* constitute about 34% and 9%, respectively, in superficial carious layer and 32% and 10%, respectively, in deep carious front.

Some culture-based studies of bacteria in dentine caries samples found that *Olsenella* and *Propionibacterium* were the most dominant in dentine caries (Munson *et al.* 2004, Schulze-Schweifing *et al.* 2014), which is similar to the results of other studies (Chhour *et al.* 2005, Wolff *et al.* 2013) which used cloning microbiological analysis for 16S rRNA bacterial identification in dentine caries. These found that *Propionibacterium* is more dominant in deep caries. *Propionibacterium* and *Olsenella* were found in 100% and 94% of samples in this study, with low abundance (0.17% and 0.24%, respectively) compared to other genera. Recently, the involvement of *Propionibacterium acidifaciens* in deep dentine caries has been demonstrated suggesting its participation in the disease process and dental caries (Aas *et al.* 2008, Lima *et al.* 2011). The role of members of the *Propionibacterium* family in degrading protein from the exposed dentine collagen matrix might be an advantage to outcompete while progressing deeper into the dentine (Chhour *et al.* 2005). Also, it has been reported before that *Propionibacterium,* an obligate anaerobe, was present inside unexposed vital pulps of teeth with deep dentine caries lesions, in addition to other anaerobes such as *Actinomyces* and *Eubacterium*, in comparison to *Streptococcus*, *Peptostreptococcus* and *Lactobacillus* (Hoshino *et al.* 1992).

In all samples, one or two to three genera were dominant, but the predominant genera differed in every patient. The pattern of distribution of dominant bacteria looks as if it is subject-specific rather than site-specific, as there was no significant difference between superficial and deep carious dentine in the abundance and prevalence of the most abundant genera. This means that the bacterial communities present in superficial and deep carious layers have a similar composition. These findings are similar to previous studies that noticed no significant differences between different layers of the carious lesion; instead, they notice considerable differences in bacterial composition among individuals (Munson *et al.* 2004, Lima *et al.* 2011, Obata *et al.* 2014).

The results show that the carious lesions can be classified according to *Lactobacillus* abundance into three distinctive clusters: low-, medium- and high-*Lactobacillus* clusters. These cluster patterns were similar in both superficial and deep carious dentine. These pattern observations agree with those observed in previous reports that used culturing and molecular methods (Hahn *et al.* 1991, Obata *et al.* 2014, Rôças *et al.* 2016). High-*Lactobacillus* clusters in superficial and deep carious dentine were characterised by high relative abundance of *Lactobacillus*, above 85% proportion of reads in about 18% and 20% of superficial and deep carious dentine samples, respectively, lower than 30% of sample that have high-*Lactobacillus* abundance (above 80% relative abundance) reported by Obata *et al.* (2014) in Japanese people. This may be because the lower limit of this cluster was different in both studies (80% versus 85%). Also in this cluster, there was associated low abundance of *Streptococcus* and other bacteria in caries lesions, which suggest that the presence of *Streptococcus* in high proportions is not necessary for caries progression in these lesions. It was reported that dental caries can occur without the presence of *Streptococcus mutans* (Beighton 2005, Gross *et al.* 2012).

In cluster 2, the microbiome was characterised by a high abundance of *Atopobium* and *Streptococcus* in addition to *Lactobacillus* in superficial and deep carious dentine;

Atopobium was introduced in 1992 as a new genus derived from *Streptococcus* and *Lactobacillus* using comparative sequence analysis (Collins and Wallbanks 1992). *Atopobium* presence in the composition of dentine caries microflora in addition to *Lactobacillus* and *Streptococcus* has been demonstrated in recent molecular studies (Aas et al. 2008, Lima et al. 2011, Obata et al. 2014). *Atopobium* is closely related to *Olsenella* (Kraatz *et al.* 2011), which results in misclassification of the V region of 16S rRNA gene between both genera (Obata *et al.* 2014). The role of *Atopobium* in dentine caries need to be investigated more in future studies.

The relative abundance of *Lactobacillus* in these two clusters (high- and medium-*Lactobacillus*) range from 30-99%. Another study, which investigated deep dentine caries samples from teeth with irreversible pulpitis and classified microbial composition according to *Lactobacillus* abundance, found that 50% of samples contained 63-96% relative abundance of *Lactobacillus* compared to the other half of samples, dominated by *Pseudoramibacter, Olsenella* and *Streptococcus* and associated with low abundance of *Lactobacillus* (Rôças *et al.* 2016). The present study reported a similar pattern of *Lactobacillus* dominance in dentine caries. The dominance of *Lactobacillus* in deep dentine carious lesions suggests that environment favours their colonisation. They are acidogenic and aciduric bacteria that produce lactic acid and create a low-pH environment that cannot be tolerated by other species (Caufield *et al.* 2015). Also, deep cavities create a low-pH environment that is suitable for the growth of *Lactobacillus* and elimination of other microbes (Takahashi and Nyvad 2011).

Lactobacillus, usually associated with tooth cavities rather than smooth surfaces (van Houte *et al.* 1972), has been proposed to be involved in the progression of caries rather than its initiation (Loesche 1986). Dentine carious lesions can provide the optimum conditions for the constant colonisation of *Lactobacillus* because they provide a low-pH, stagnant habitat, anaerobic environment and a continuous source of carbohydrates (Caufield *et al.* 2015). Therefore *Lactobacillus* was only detectable in the caries-active subject but could not be detected in caries-free subjects (Yang *et al.* 2010, Piwat *et al.* 2010).

In cluster 3, *Lactobacillus* was very low or absent. Instead, samples were dominated by *Atopobium*, *Streptococcus*, *Prevotella*, *Pseudoramibacter*, *Peptostreptococcus* and *Rothia*, suggesting that members of these genera will be universally present but compete with *Lactobacilli* within the lesion. It was noticed that in deep carious dentine samples 15 and 23 in cluster 3, *Pseudoramibacter* was the predominant genera when there are low abundances of *Atopobium* and *Streptococcus*, suggesting its competition with other genera on the deeper aspect of a carious lesion. These observations were similar to those of another study investigating the bacterial composition of deep carious lesions associated with irreversible pulpitis symptoms, where the authors found *Pseudoramibacter* was the predominant genus in two samples associated with low abundance of *Lactobacillus* (Rôças *et al.* 2016). *Pseudoramibacter* is a gram-positive obligatory anaerobic bacterial genus. It is believed that they play a role in the infection of root canal space. It was reported that *Pseudoramibacter* was one of the most abundant and prevalent bacteria in chronic root canal infections (Siqueira *et al.* 2009, Santos *et al.* 2011). Also, *Peptostreptococcus* observed in cluster 3, which is anaerobic gram-positive bacteria, was reported to be one of the most predominant bacteria in acute root canal infections (Santos *et al.* 2011).

The shift of *Lactobacillus* abundance in the different lesions can be explained on various bases. Firstly, it can be explained as a transition from a cariogenic microbiome to an infectious microbiome of dental pulp; especially that the other observed non-*Lactobacillus* taxa in dentine caries were frequently detected in root canal infections

(Siqueira and Rôças 2004, Santos *et al.* 2011). Secondly, there is a shift in the ecology of carious dentine because of changes in dietary supply, oxygen conditions and pH fluctuations. In addition, the inflammation of the pulp can have an impact on the microbiome of carious dentine by providing a protein dietary supply, such as glycoproteins from inflammatory exudate (Rôças *et al.* 2015). All these factors can participate in the observed shift of microbiome of dentine caries from high-*Lactobacillus* to medium- and low-*Lactobacillus* with associated dominance by other types of bacteria.

It was reported previously that there are no significant differences in microflora between superficial, middle carious dentine layer and advancing front of the lesion except for minor differences (Munson *et al.* 2004, Lima *et al.* 2011). These studies used earlier molecular techniques such as clonal analysis and reverse-capture checkerboard hybridisation assay, respectively, for detecting a specific number of predominant species in dental caries. Also, results from this study show that there is no significant difference between superficial and deep layers of carious dentine in the abundance and prevalence of major bacterial genera (above 0.01% relative abundance). However, there were some bacteria (<0.01% abundance) present in the superficial carious layer of dentine but not in the deeper layers and vice versa. These differences between layers of carious dentine were small compared to greater differences in composition of carious lesions between subjects, which is similar to the conclusion of a previous study that reported similar inter-individual variations in microbiota of deep dentine caries, compared to small variation in composition at various depths of the lesions (Obata *et al.* 2014).

In terms of the relation between clinical features and bacterial composition of the carious lesions, it was observed that there was a statistically significant association between some bacterial genera and the clinical features of the investigated lesions. However, these microbes were present in low abundances (<few reads), therefore, it was difficult to confirm the role and source of these bacteria, if it was from a nosocomial source or an evolving part of dentine caries microbiome ecology. However, their role in histopathological changes of pulp inflammation/infection needs to be confirmed in future studies.

In superficial carious dentine samples, *Caloramator, Gluconobacter* and *Dermacoccus* were more prevalent in severe cases compared to mild cases. *Dermacoccus* was isolated from human skin and gastrointestinal tract of vertebrae; the first reported infection with *Dermacoccus* in human was in catheter-related bloodstream infection (Takahashi *et al.* 2015). *Oxalobacter* and *Acholeplasma* were associated with failure of treatment; *Oxalobacter* is a gram-positive anaerobic bacterium, first isolated from anoxic freshwater sediments in 1989 (Dehning and Schink 1989). *Oxalobacter* genus in the family *Oxalobacterecea* is the only strictly anaerobic genus in this family. These bacteria are usually found in water, soil and plants, but some members are opportunistic human pathogens (Baldani *et al.* 2014).

In deep carious dentine samples, *Paraprevotella, Stenoxybacter, Elizabethkingia, Coriobacterium, Gallibacterium, Actinoplanes, Uliginosibacterium, Vogesella, Tolumonas, Thiobacillus, Roseospira, Deferribacter, Bartonella, Ralstonia, Limnobacter, Propionicimonas* and *Runella* were significantly more prevalent in failed cases compared to successful cases. *Elizabethkingia* causes outbreaks of meningitis in premature newborns and infants in neonatal intensive care units. The bacterium is also a rare cause of nosocomial pneumonia, endocarditis, postoperative bacteraemia, and meningitis in immunocompromised adults and recently was found in a case of a fatal necrotising fasciitis in diabetic patients (Lee *et al.* 2006). *Dermacoccus* and

Herminiimonas were more prevalent in severe cases compared to mild cases. It appears that the presence of *Dermacoccus* in both superficial and deep carious dentine samples usually associated with cases with severe symptoms compared to its absence in cases with mild symptoms.

It was observed that the average relative abundance of some bacteria such as *Atopobium, Pseudoramibacter, Parascardovia, Neisseria, Actinomyces, Corynebacterium, Fusobacterium, Porphyromonas* and *Peptostreptococcus* in deep carious dentine was higher in the failed teeth compared to the successful teeth, suggesting that their presence in higher proportions in the deeper layer of carious lesion can predict failure of the treatment. Most of these bacteria have been isolated from infected root canal space (Santos *et al.* 2011) and from deep carious lesions with irreversible symptoms (Rôças *et al.* 2016). However, in the analysis of symptoms intensity, it was noticed that these microbes tended to show lower proportions in cases presented with severe symptoms compared to cases with mild symptoms (Figure 6-30), and in the scope of the subjectivity of both symptoms presentation and clinical diagnostic investigations, this suggested that they have a role in initiating asymptomatic irreversible pulpitis (Torabinejad and Shabahang 2015). In contrast, it was observed that in 60% of samples (samples 4, 7 and 14 in the superficial layer; samples 1, 5 and 13 in the deep layer – see Figure 6-10 and Figure 6-11) in cases with severe symptoms, *Lactobacillus* was the most abundant genus (35-97% relative abundance). This was confirmed by PCA and average relative abundance analysis of bacterial taxa according to symptoms severity, which showed higher proportions of *Lactobacillus* and *Prevotella* in deep carious samples of severe symptoms cases. This is in agreement with another study which found that *Lactobacillus* in deep carious lesions can be associated with a continuous pain in teeth with irreversible pulpitis (Rôças *et al.* 2015).

The results from this study indicated that bacterial flora in remaining dentine after caries excavation exhibited a varied relative abundance of bacterial taxa between the minimally invasive and the control. This may suggest that one protocol leaves a different dentine microbiome from the other; however, average relative abundance does not reflect the infectivity of the remaining dentine, which is the total number of viable / non-viable bacterial cells in mg of wet dentine. Results from qPCR show that there was no significant difference between the two methods in reducing bacterial count. Observed different bacterial prints of the remaining carious dentine between the two groups may refer to the varied ecological effect of each excavation process on the microbiome of the remaining dentine after excavation. The mechanical and chemical differences between the two excavation methods (rotary cutting versus abrading) may mechanically force some microorganisms from superficial to deep carious dentine layer. In addition, chemical stresses can have an impact on the bacterial print. There were previous reports suggesting a shift in the phenotypic and genotypic diversity of the remaining dentine after various restorative treatments (Paddick *et al.* 2005, Rupf *et al.* 2008, Hedenbjörk Lager 2014).

There was inter-individual variation compared to smaller variations in bacterial community composition among different depths in the same lesion. It has been observed that there was a specific configuration of the dominance of *Lactobacillus* in most selected lesions. Early invasion of the dentine matrix from supragingival plaque and / or dietary and salivary microbiota probably results in the dominance of specific species in a later stage. Eventually, lesion progression depends on the availability and abundance of aciduric and acidogenic bacteria initially followed by the migration of asaccharolytic and proteolytic bacteria that could elicit additional degradation of the dentine matrix and pulpal degeneration. Therefore, early events in carious lesions could determine bacterial pathogenicity and composition of endodontic infection microflora, finally.

6.6.3 Conclusions:

1- There was no significant difference between the two excavation techniques in reducing bacterial loads in deep carious lesions. Therefore, the first null hypothesis is accepted.

2- The top three most abundant genera in dentine carious lesions were *Lactobacillus, Atopobium* and *Streptococcus*. The most prevalent genera in the samples were *Streptococcus, Prevotella* and *Atopobium.*

3- There was no significant difference in abundance and prevalence of bacterial taxa between superficial and deep carious dentine samples. Therefore the second null hypothesis is accepted.

4- There was a significant association between the presence of some bacterial taxa and clinical features associated with dentine carious lesions. Therefore the third hypothesis was rejected.

5- Regarding heterogeneity of phylogenetic distribution in deep carious lesions, it was noted that carious lesions can be classified according to the relative abundance of *Lactobacillus* species. Carious lesions with low-*Lactobacillus* abundance were dominated by other bacterial taxa which were frequently isolated from root canal infections. This suggests that the source of pulpal / endodontic infection arises from deep carious microbiome.

6- The present observations confirm the diversity of the bacterial community in terms of dominance in a specific lesion inter-individually, which suggest potential early or late conditions that drive dominance of specific species in the carious lesion and subsequently infected pulp space. It seems that the shift in microbiota during caries advance to the pulp indicates coexistence of caries and root canal pathogens.

7- The knowledge acquired from identification of bacterial taxa associated with deep dentine caries can help identify the possible predisposing bacteria of initial pulp infection and may help develop therapeutic strategies for minimally invasive treatment of symptomatic teeth, with more predictable outcome to prevent de-vitalisation of teeth.

Chapter 7 General Summary and Suggestions for Future Work

7.1 Summary

The aim of the randomised controlled clinical trial was to provide an evidence base required to justify the use of minimally invasive caries excavation in the treatment of teeth with deep carious lesions in patients presenting with signs and symptoms of reversible pulpitis, as opposed to conventional caries excavation.

Treatment of deep carious lesions in symptomatic teeth is often associated with diagnostic and clinical challenges. These are represented by the subjectivity of clinical diagnostic tests and the limited amount of diagnostic information provided by conventional two-dimensional, often geometrically distorted periapical radiographs,which are unable to overcome the noise produced by superimposed anatomical structures such as the cortical plate of the mandible or the zygomatic arch (S. Patel *et al.* 2012). In order to overcome these drawbacks CBCT was used to help identify teeth that presented with initial periapical lesions. Such teeth were excluded from the study population before treatment, to reduce the probability of including teeth with irreversible pulp changes, as there is currently no reliable method of identifying objectively the extent of pulp inflammation.

It was found that single-visit treatment of teeth with deep carious lesions had a better outcome when compared to two-visit treatments (Schwendicke *et al.* 2013a). These teeth need to be restored with direct resin composite restorations to provide mechanical strength, leakage resistance, wear resistance, and improved aesthetics of the definitive restoration overlying the indirect pulp capping materials. In this trial, MTA was used, which has weak mechanical properties that may hinder its immediate use with other restorative materials usually used for direct restorations, such as resin composite. A mechanical interaction of MTA with the overlying restorative materials was assessed *in vitro* prior to commencing the trial, to evaluate the durability of the bond between MTA and GIC or resin composite at different time intervals of MTA setting.

Excavation of caries in deep dentine carious lesions using chemo-mechanical caries removal results in retaining more caries-affected dentine compared to excavation with conventional rotary dental burs, because the latter tend to remove more tooth structure due to the lack of self-limiting capacity and tactile sensation. Remineralisation and hardening of this carious dentine tissue are of a clinical significance to enhance mechanical integrity and performance of the restoration. An *in-vitro* study was conducted to evaluate the mineral deposition and mechanical hardening of remaining caries-affected dentine after excavation with Carisolv™ gel or dental burs, and to compare the invasiveness of both tools in managing dentine caries in terms of preserving healthy tooth structure.

Dentine carious lesions harbour complex communities of bacteria that are considered to be the main causative agent of pulp infection. It is important to identify phylogenetic profiles of microbiota associated with deep dentine carious lesions at different levels within the lesion, to identify the shift in bacterial activity and virulence and its correlation with clinical presentation and outcome. A non-culture-based microbiological analysis of superficial and deep dentine caries samples collected from the clinical trial population was conducted to identify the amount and composition of microbiota associated with superficial and deep layers of carious dentine.

In the clinical trial, the outcome of teeth after one year of treatment was assessed clinically and radiographically using PA and CBCT. CBCT was able to detect periapical lesions in teeth presenting with signs and symptoms of reversible pulpitis, consistent with other studies that confirm the superior performance of CBCT compared to PA radiographs in detecting periapical lesions (Patel *et al.* 2009, S. Patel *et al.* 2012, Hashem *et al.* 2015).

In the clinical trial, direct etching with phosphoric acid and direct placement of resin composite over freshly mixed MTA was avoided. Instead, a layer of GIC was placed over partially set MTA before restoration with resin composite at the same appointment. This was based on the conclusions acquired from the *in-vitro* study of the bond strength between MTA and resin composite or GIC. The clinical relevance of the results showed the importance of timing of resin composite placement over MTA. There was low mechanical interlocking between MTA and resin composite after 10 minutes of MTA mixing, which resulted in low bond strength between them compared to that yielded between MTA and GIC or to that obtained between MTA and resin composite after 24 hrs, 72 hrs and 30 days of MTA setting time (Ali *et al.* 2016).

Clinical and radiographic results of the clinical trial showed more favourable outcomes in teeth treated with the minimally invasive protocol compared to the control group. The use of Carisolv^{TM} gel and an operating microscope can help preserve the health of the pulp of symptomatic teeth presenting with deep carious lesions, compared to the use of rotary burs without magnification. Minimally invasive protocols help in reducing pulp symptoms in cases with more severe symptoms. Comparison of results of this trial with the results of another clinical trial in symptomatic teeth presenting with deep carious lesions (Hashem *et al.* 2015), which used similar clinical and radiographic settings including Carisolv^{TM} gel excavation and pre- and post-operative CBCT scans, showed that the use of magnification in deep caries excavation in this trial produced better results than Carisolv^{TM} gel alone. However, long-term follow-up of these cases is needed to monitor pulp and restorative outcomes of both treatments.

Teeth excavated with Carisolv^{TM} gel and capped with MTA *in vitro* showed mineral deposition and hardening in caries-affected dentine up to the level of sound dentine after a storage period. Raman spectroscopy and Knoop microhardness tests were used to assess the mineral levels and microhardness before and after remineralisation. Clinically, remineralisation of caries-affected dentine after minimally invasive caries excavation helped arrest caries progression and protected the pulp, with the additional advantage of preserving tooth structure.

In addition to the evidence of remineralisation of caries-affected dentine after chemo-mechanical excavation *in vitro*, this thesis provided *in-vivo* evidence of the similar ability of chemo-mechanical and mechanical-only caries excavation to reduce the number of remaining bacteria in deep carious cavities. This study proved that some microorganisms persist after the process of caries excavation, irrespective of the method of excavation, without necessarily affecting pulpal sensibility and/or restoration integrity at one-year recalls.

Furthermore, results showed that there is substantial microbial variation among carious lesions. In fact, a particular micro-ecosystem was associated with each lesion, depending on local environment and clinical conditions. This concurs with the ecological microbial hypothesis, which states that disease promotion is caused by an unbalanced ecosystem resulting from the adaptation of ecosystem to changes in the oral environment. The abundant variation of microbiota in dentine caries from the results of this and previous studies suggest that the body of local microorganisms needs to be considered as contributing to initiation and progression of dentine caries, rather than considering individual caries-promoting species only (Gross *et al.* 2010).

In conclusion, this thesis provided laboratory, clinical, radiographic and microbiological evidence for the improved performance of the minimally invasive approach in the treatment of deep carious lesions in symptomatic teeth compared to a conventional approach.

7.2 Suggestions for future work

1- Longer term follow-up of patients in the clinical trial. This will include:

a- Clinical and radiographic assessment of patients for a period of 3-5 years, which involve using CBCT scans to assess both the pulp sensibility and periapical status of teeth in the long-term follow-up.

b- Long-term assessment of the performance of the overlying restoration as a one-year follow-up is not sufficient to observe the performance of the resin composite restorations. Restorative failure can play a significant role in affecting the success/failure rates of the vital pulp therapies.

2- Collecting further microbiological samples from carious dentine and root canals (via paper points during access opening for root canal treatment) from the treated teeth that could fail clinically/radiographically in future follow-ups of this trial. Data acquired can help to identify the transition of microbial flora associated with irreversible pulpal inflammation and/or primary root canal infection in the same individuals.

3- Three-dimensional assessment of dentine deposition of the treated teeth using multi-dimensional radiographic modality of CBCT to detect the change in the pulp size and geometry after one or two years of treatment. Pulp space restriction and/or pulp calcifications could present future challenges for root canal treatment.

4- Incorporation of Doppler flowmetry technique in the diagnosis of symptomatic teeth presenting with signs and symptoms of reversible pulpitis could provide a more objective indication of pulpal vitality in these teeth. A study including CBCT and Doppler flowmetry can help to reduce diagnostic shortcomings associated with periapical radiographs and thermal/electrical pulp testers in the diagnosis of symptomatic teeth.

Appendices

Appendix 1: Published papers

Ali, A., Banerjee, A. and Mannocci, F. 2016. Effect of adhesive materials on shear bond strength of a mineral trioxide aggregate. American journal of dentistry, 29 (1), pp.46-50.

Research Article

Effect of adhesive materials on shear bond strength of a mineral trioxide aggregate

AHMED ALI, BDS, MSc, AVIJIT BANERJEE, BDS, MSc, PhD, FDS & FRANCESCO MANNOCCI, MD, DDS, PhD

ABSTRACT: Purpose: To compare the shear bond strength (SBS) and fractography between mineral trioxide aggregate (MTA) and glass-ionomer cement (GIC) or resin composite (RC) after varying MTA setting time intervals. **Methods:** MTA was mixed and packed into standardized cavities (4 mm diameter × 3 mm depth) in acrylic blocks. RC with 37% H_3PO_4 and type 2 (etch and rinse) adhesive, or conventional GIC was bonded to the exposed MTA sample surfaces after 10-minute, 24-hour, 72-hour and 30-day MTA setting intervals (n=10/group, eight groups). Samples were stored (37°C, 24 hours, 100% humidity) before SBS testing and statistical analysis (ANOVA, Tukey LSD, $P< 0.05$). Fractography was undertaken using stereomicroscopy for all samples and three random samples/group by using SEM. **Results:** Significant differences between all groups were found ($P= 0.002$). SBS of RC:MTA (Max 5.09±1.79 MPa) was higher than the SBS of GIC:MTA (Max 3.74 ± 0.70 MPa) in 24-hour, 72-hour and 30-day groups except in the 10-minute MTA setting time groups, where SBS of GIC:MTA was higher. There was a significant effect of time on SBS of RC: MTA ($P= 0.008$) and no effect on SBS of GIC:MTA ($P= 3.00$). Fractography revealed mixed (adhesive/cohesive) failures in all groups; in RC:MTA groups there was a decrease in adhesive failure with time in contrast to the GIC:MTA groups. (*Am J Dent* 2016;29:46-50).

CLINICAL SIGNIFICANCE: Placement of resin composite over partially set MTA directly might result in low micro-mechanical and/or chemical interaction between the two materials.

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Introduction

Direct pulp capping is a procedure where biocompatible therapeutic materials like calcium hydroxide or mineral trioxide aggregate (MTA) is placed on pulp tissue that has been in danger of caries, trauma or restorative procedures. Its purpose is to provide a sealed barrier over the exposed pulp, preventing bacterial leakage and stimulating the formation of a histological dentin bridge, all to aid the continued preservation of pulp vitality.[1] The bond and seal between the restorative material and pulp capping agent is important as well as that between pulp capping and dentin.[2] If there is an inadequate bond or seal, ingress of bacteria into the pulp and ultimate failure of the pulp capping procedure will occur.[3-6]

MTA is a hydrophilic cement composed of calcium oxide, silica and bismuth oxide.[7] It has weak mechanical properties that preclude its sole use as a definitive restoration at the same appointment, while still providing a seal and environment that enhances dentin-pulp complex healing.[8] MTA has a reported setting time of between 75 minutes up to 73 hours.[9] Therefore it has been used in conjunction with other restorative materials to provide a physico-mechanical barrier until maturation. This requires a two-step clinical procedure as there will be need to restore the tooth with a permanent RC restoration later.[10]

Recently investigations on deep caries management have recommended a direct seal in one visit after selective caries removal.[11,12] If the definitive restoration is placed directly after minimally invasive selective caries removal, rather than following a sequential procedure, this can result in an improved preservation of pulp vitality.[13] Therefore, the direct placement of resin composite over the MTA pulp cap can provide a simple, reliable operative technique to restore the pulp-capped tooth with a definitive restoration without the need for a temporary/provisional restoration.

In vitro tests are not a substitute for clinical studies to assess outcome success of restorations; however, understanding the mechanical nature of the interface between different restorative materials in laminate restorations with MTA provides the evidence base to optimize longevity of such restorations.

The shear bond strength test was chosen for this study in order to obtain viable data from mechanically weak sample materials and to allow comparison with other previously published studies.[2,14-17] This in vitro study evaluated the shear bond strength (SBS) between white MTA and glass-ionomer cement (GIC) or resin composite (RC) after 10 minutes, 24 hours, 72 hours and 30 days intervals of MTA setting time. It also assessed the fractography or interfacial failure mode (IFM) between the MTA and RC or GIC. The null hypotheses were that (1) there was no difference in shear bond strength between MTA and RC or GIC; (2) that there was no effect of the setting time period of MTA on the shear bond strength with RC and GIC; and (3) that there was no difference between RC and GIC in terms of fractographical analysis to MTA.

Materials and Methods

Eighty acrylic resin blocks were prepared with 4 mm diameter and 3 mm deep standardized cavities ready for the MTA placement. Plastic cylindrical transparent tubes with dimensions of 3.2 mm diameter by 3 mm height were prepared to act as molds for the RC or GIC restorative material. MTA applicaps (Acteon[a]) were activated and triturated in an amalgamator (Ultramat 2[b]) following the manufacturer's instructions. The MTA was extruded into the acrylic cavities using a delivery plunger (No. 01/98[c]), and condensed with an appropriate condenser (No. 04/6[d]). A cotton pellet or a paper point was used to remove the extra moisture to prevent over hydration as per the manufacturer's instructions. Eight groups

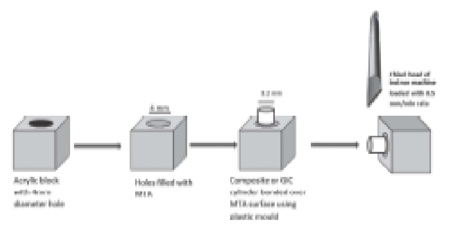

Fig. 1. Schematic diagram illustrating sample preparation and shear bond strength testing.

Table 1. Chemical composition of materials used in the study.

Material	Material composition
White mineral trioxide aggregate - MTA cap	Powder: mixing of mineral oxides based on calcium and tungstate. Liquid: water
GIC - Fuji IX GP	Powder: fluoro-alumino-silicate glass, polyacrylic acid powder Liquid: polyacrylic acid, polybasic carboxylic acid
RC - N'Durance	The resin based matrix contains approximately 19 wt % of ethoxylated BisGMA, UDMA and dicarbamate dimethacrylate dimer acid. The filler system contains approx. 80 wt% (65 vol%) silanated 40 nm ytterbium fluoride, silanated 500 nm barium glass and 10 nm silica. There is approximately 1 wt% of catalyst, inhibitors and pigments.
Etch and rinse adhesive - XP Bond	Carboxylic acid modified dimethacrylate (TCB resin) Phosphoric acid modified acrylate resin (PENTA) Urethane dimethacrylate (UDMA) Triethyleneglycol dimethacrylate (TEGDMA) Hydroxyethylmethacrylate (HEMA) Butylated benzenediol (stabilizer) Ethyl-4 dimethylaminobenzoate Camphorquinone Functionalized amorphous silica, t-butanol
Etchant - Etch Gel	40% phosphoric acid

(n=10/group) MTA samples were left to set in 37°C and 100% humidity conditions, before bonding to either GIC (Fuji IX[e]) or RC (N'Durance[f]).

In four experimental groups (1, 3, 5 and 7, n=10/group) RC bonded to MTA after 10 minutes, 24 hours, 72 hours and 30 days setting times respectively (n=10). These times were chosen to simulate clinical situations. Also this was done to assess the effect of degree of MTA maturation on the bond to restorative materials, where 10 minutes represent the initial clinical setting time suggested by the manufacturer, 24-72 hours represent the complete setting reaction of the cement and 30 days represent a possible clinical situation for placement of permanent restoration especially in case of vital pulp therapy. The set MTA samples were etched with 37% phosphoric acid gel (Etch Gel[g]) for 15 seconds, rinsed with water for 30 seconds and dried using an oil-free air syringe for 5 seconds. A type 218 (etch and rinse) adhesive (XP Bond[h]) was agitated onto the surface of samples with a clean micro-brush. Two layers of adhesive were applied before a mild air syringe flow was applied for 10 seconds to evaporate all the solvent. The adhesive was light-cured for 20 seconds (Optilux 501[i]) with an output wavelength range of 400-505 nm and output light intensity of 850 mW/cm². The transparent plastic molds were filled with RC and placed on the surface of MTA samples. The composite was condensed and light-cured for 40 seconds from the top plus 40 seconds from the sides and a sharp scalpel blade was used to cut through and remove the plastic tubes. Figure 1 shows the preparation of the specimens and shear bond strength testing.

In the remaining four experimental groups of MTA that were bonded with GIC (Groups 2, 4, 6 and 8, n=10/group), the GIC powder and liquid (shade A2) were mixed on a mixing pad with a plastic spatula as per the manufacturer's instructions, and placed over the center of the MTA surface by packing the material into the cylindrical plastic tubes. GIC conditioner was not used over the MTA surface because there was no dentin smear layer to be removed before GIC placement as in the case of the prepared tooth surface. The specimens were allowed to set for 10 minutes within the plastic tubes to ensure completion of the initial setting reaction of the GIC. The plastic tubes were removed with a sharp scalpel as previously described. Samples were stored in distilled water (as in other studies[2,16,19]) at 37°C and 100% humidity for 24 hours. All samples were prepared by the same operator. Chemical compositions of materials used in this study are summarized in Table 1.

Shear bond strength testing (SBS) - Each specimen was mounted in a universal testing machine (Model 5569 A[j]). Bond failure between MTA and RC or GIC in each specimen was obtained by using a knife-edged blade applied with a crosshead speed of 0.5 mm/minute. The values were recorded in Newtons and transformed to MPa by dividing the peak load at failure by the interfacial surface area of the GIC/RC and MTA.

Fractography - The interfacial failure modes (IFM) between MTA and RC/GIC were assessed optically using stereo-microscopy[k] with ×4.5 magnification by assessing both the MTA surface and the RC or GIC surfaces.

The modes of failure were categorized as follows:

a. Adhesive failure between MTA and GIC.
b. Cohesive failure within MTA.
c. Cohesive failure within RC or GIC.
d. Cohesive failure within adhesive.
e. Adhesive failure between adhesive and MTA.

Each mode is presented as a percentage for each specific group and because many samples had combined or mixed modes of failure (i.e. cohesive and adhesive), each mode was added to the percentage of its own category.

SEM analysis - A scanning electron microscope[l] was used to examine the ultrastructure of the interface surfaces (accelerating voltage of 3.5 and 10 kV, working distance of 10 mm, magnifications: ×500, ×2,000 and ×2,500 under high vacuum conditions). Three randomly selected samples per experimental group were examined, gold sputter-coated before SEM analysis (Emitech K550[m]). Two samples were used to assess the interface between MTA and RC or GIC longitudinally and one sample was used to scan part of interface surface horizontally.

Statistical analysis - The outcome variable for this study was the shear bond strength value. The means and standard deviations of shear bond strength for all groups were calculated. Data analysis showed normal distribution of data using histograms; Shapiro-Wilk and Kolmogorov-Smirnov tests and the mean SBS for all groups were compared using one-way

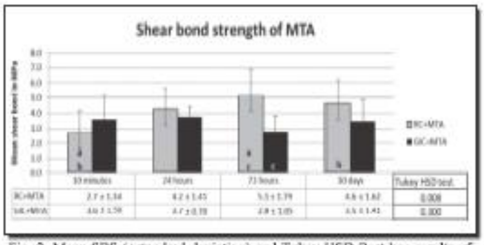

Fig. 2. Mean SBS (±standard deviation) and Tukey HSD Post-hoc results of all experimental groups. Different superscript letters indicate statistically significant differences ($P< 0.05$).

Table 2. Distribution of modes of failure and percentages recorded in all experimental groups. MTA, mineral trioxide aggregate; RC, resin composite; GIC, glass-ionomer cement.

Modes of Failures in RC/MTA	10 MINUTES	24 HOURS	72 DAYS	30 DAYS	trend
b= Cohesive within MTA	90%	90%	90%	90%	
d= Cohesive within Bonding agent	0%	0%	60%	0%	
e= Adhesive between bonding agent/MTA	9.5%	9.5%	30%	10%	
c= Cohesive within RC	0%	0%	0%	0%	
Modes of Failures in GIC/MTA	**10 MINUTES**	**24 HOURS**	**72 DAYS**	**30 DAYS**	**trend**
a= Adhesive between MTA and GIC	70%	80%	80%	60%	
b= Cohesive within MTA	40%	70%	70%	50%	
c= Cohesive within GIC	0%	20%	0%	0%	

ANOVA. Tukey LSD Post-hoc test was used and the level of statistical significance was set at $P= 0.05$. Also, Tukey Post-hoc test was used to assess the effect of timing intervals of MTA setting on the mean SBS with RC and GIC. All statistics were performed using IBM SPSS[a] statistics (version 20) software.

Results

Shear bond strength - Figure 2 shows the mean SBS (±SD) values. The highest mean SBS (±SD) of MTA:RC was observed in the 72-hour group (5.09 ± 1.79 MPa) and the highest mean SBS of MTA:GIC in the 24-hour group (3.74 ± 0.70 MPa). There were no pre-test failures in any of the experimental groups. ANOVA used for comparison between all experimental groups revealed highly significant differences between all groups ($P= 0.002$). Mean SBS of MTA:RC was higher than mean SBS between MTA:GIC in the 24-hour, 72-hour and 30-day groups respectively except in the 10-minute groups the mean SBS of MTA:GIC was higher than SBS of MTA:RC.

Timing effect - Tukey LSD post-hoc test showed that there was a statistically significant effect of the MTA setting time (10 minutes, 24 hours, 72 hours and 30 days) on the SBS of MTA:RC ($P= 0.008$) and there was no statistically significant effect of timing on the SBS of MTA:GIC ($P= 0.300$; Fig. 2).

Fractographical analysis - Assessment of interface failure modes (IFM) revealed a high rate of cohesive failures within the substrate material (MTA) in all groups (category B). Conversely, there were a low number of cohesive failures observed within both RC or GIC (category C) compared to those occurring in MTA as shown in Table 2. All groups showed combined (cohesive and adhesive) failures. In the RC groups, there was both cohesive failure in MTA and adhesive failure between MTA and bonding agent. In the GIC groups there was both cohesive failure in MTA and adhesive failure between MTA and GIC as shown in Table 2. Stereomicroscopic images of the modes of failure are shown in Fig. 3.

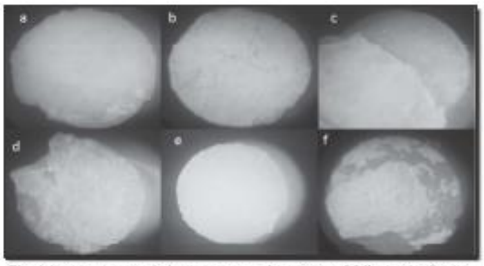

Fig. 3. Stereomicroscopic images representing modes of failure, (a) adhesive failure MTA/GIC, (b) cohesive failure in MTA, (c) cohesive failure in GIC, (d) cohesive failure in bonding agent, (e) adhesive failure in bonding agent, (f) mixed cohesive/adhesive failures presented as two separated modes.

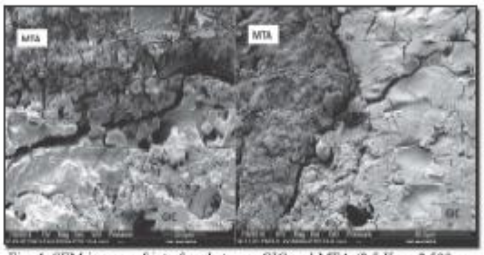

Fig. 4. SEM images of interface between GIC and MTA (3.5 Kv, ×2,500 on left and ×2,000 on right). Arrows show the cracks in the GIC layer.

Scanning electron microscopy - The scanning electron microscopy analysis of the interface between MTA and GIC showed vertical and horizontal cracks in the GIC around accumulations of polyacrylic acid associated with gaps between the two materials. These were limited to the superficial interfacial layer of GIC with MTA (Fig. 4). RC-MTA interface analysis revealed a continuous interface with fewer gaps (Fig. 5) and filamentous network of adhesive infiltrating the void spaces between the MTA particles forming a mesh-like structure after adhesive failure (Fig. 6).

Discussion

The bond between the MTA and RC or GIC is as important as the seal between MTA and tooth structure for the overall treatment success. Adhesive materials like RC provide a better seal than GIC in coronal restorations; if it can provide a good bond with MTA, this can increase durability of vital pulp therapy restorations. Proper bonding of RCs to pulp capping biomaterials produced even distribution of stresses over the adhesive layer between the two materials.[7] The most common method to evaluate the adhesive properties of restorative materials is their bond strength assessment. Therefore, the shear bond strength test was chosen for this study, complementing other published studies on MTA.[2,14,16,17,20,21]

Some studies have suggested using RC directly over partially set MTA, while others have proposed layering the freshly mixed MTA with GIC after waiting 45 minutes.[17,22] In

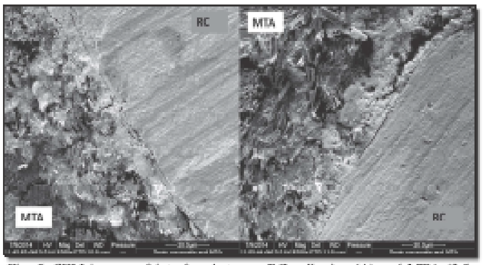

Fig. 5. SEM images of interface between RC, adhesive (A) and MTA (3.5 kV, ×2,500).

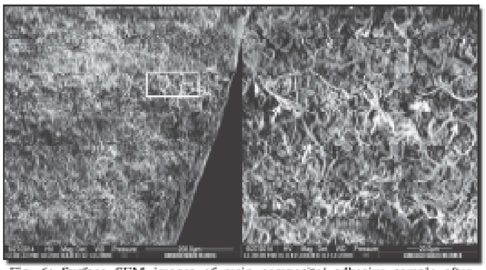

Fig. 6: Surface SEM images of resin composite/ adhesive sample after adhesive failure. Arrows show filaments of bonding agent resin infiltrated into MTA spaces after adhesive failure, with 10.0 kV, x500 and x2500 magnification.

the present study, two factors affected the SBS to MTA: the type of restorative material used and the setting time of MTA before bonding. The bond of RC to partially set MTA was weaker than that of GIC to partially set MTA, however it was higher in later setting time groups. Also, there was no statistically significant effect of MTA setting time on the SBS to GIC. While there was a statistically significant effect of MTA setting time on SBS to RC (Fig. 2). Therefore, layering of MTA with RC is time-dependent and non-time dependent in case of layering with GIC.

GICs bind to dental hard tissues via a physicochemical process involving ionic exchange between the interfacial substrates. The phosphate ions in the tooth structure are replaced by carboxyl groups of GIC and the latter form ionic bonds with calcium ions of dental hydroxyapatite.[23] The shear bond strengths of conventional GICs to enamel and dentin or metals are relatively low, ranging from 3-7 MPa.[24-26] In the context of this study, the SBS of MTA:GIC ranged from 2-5 MPa. The possible interfacial reactions between GIC and MTA include the carboxyl group of the polyacrylic acid interacting with the calcium of the MTA to form calcium salt complexes. The formation of calcium salts in the interface was not affected by the MTA setting time intervals.[27] However it was affected by the condition of moisture (dry or wet setting condition).[28] An alternative hypothesis of GIC:MTA interaction is through a by-product formation by condensation of silicate hydrate of MTA with GIC.[27]

The first null hypothesis was rejected because the mean SBS of MTA:RC was higher than the mean SBS of MTA: GIC except in the earliest setting groups (10 minutes). The higher exhibited shear bond strength to RC may be explained by the fact that the phosphoric acid etch provides a clean surface with an etched honeycomb pattern increasing the micromechanical attachment. Kayahan *et al*[19] proposed that surface gel-like amorphous structures and needle-like crystals in MTA are eroded during acid etching.[19] This selective removal of matrix from the periphery of the crystals without a significant loss of MTA structure may produce an ideal surface for bonding with RC by infiltration of bonding agent into these spaces forming a filamentous network.[2,7,19,21]

In the present study, the RC was bonded using an etch and rinse (type 2)[18] adhesive system rather than a self-etch (type 3 or 4)[18] adhesive because the findings from previous studies[14,16,20] have demonstrated that total etch adhesives performed better than one-step self-etching adhesives when used to bond RC or compomer to MTA. The lower SBS of MTA:RC compared to that of MTA:GIC in the 10-minute setting groups may be attributed to the fact that the water used to rinse away the phosphoric acid etchant before application of the adhesive could have altered the microstructure of the MTA.[29] This finding complements those of studies[16,19] which found acid-etch procedures to deleteriously affect the compressive strength and surface microhardness of MTA and therefore advised postponing acid etching of MTA until 96 hours after MTA mixing to permit the material to reach its ideal physical properties. However, other studies[17] found higher SBS of RC when bonded immediately to MTA compared to 45-minute and 24-hour intervals. They suggested that the MTA was more porous during the initial setting stage and the adhesive was able to penetrate more deeply into the MTA surface leading to a stronger initial bond formation.[17]

Also, the second null hypothesis was rejected because there was a statistically significant effect of MTA setting time on the SBS of MTA:RC ($P= 0.008$). In contrast, there was no statistically significant effect of MTA setting time on SBS between MTA:GIC ($P= 0.300$). This is in agreement with other studies[15,16] reporting similar mean shear bond strengths between GIC and MTA after 45 minutes and 72 hours setting time of MTA. During MTA preparation, different liquids can affect its physical properties and setting time.[30] The critical role of water in the setting of MTA has already been shown.[31] It is believed that there is a detrimental effect of placing GIC over unset MTA, in that GIC will absorb water and lead to incomplete hydration and increased porosity of MTA.[32] However other studies concluded that the effect of GIC placement over MTA after different time intervals and setting conditions was transient.[22,28] As the interaction between GIC and MTA may be mediated by the formation of calcium salts, similar to interaction of GIC with the tooth structure, it is assumed that the setting of GIC is not hindered by its combination with MTA.[27] Also, the GIC and deeper layers of MTA did not seem to be affected.[22,28]

The most common mode of failure was cohesive within the more brittle and weak MTA substrate; however the third null hypothesis was rejected as there was a notable increase in adhesive mode of failure between GIC and MTA with time in contrast to the RC trend of decrease in adhesive failures by time (Table 2). The fractographical analysis results coincide with the results of SBS, as the bond between RC and MTA increased; when more time was given for MTA to set there were less adhesive failures between RC and MTA.

One of the limitations of the present study was the use of the shear bond strength test which may not simulate fully the various natural stresses on substrate materials in teeth treated with vital pulp therapy. Natural, synthetic saliva or phosphate buffered saline was not used for storing the samples which may influence the bond and seal between different materials of the study, However, in actual clinical situations, MTA might be exposed to distilled water or saline from the coronal side as a means of moisture supply for the setting reaction.[33] Clinical studies can assess the relative success and outcome of any bond between two materials. However, in vitro studies are often the preferred method of measuring bond performance as they afford data rapidly and are less difficult to execute. In conclusion, shear bond strengths between RC and MTA were significantly higher than that of GIC and MTA after 24 hours, 72 hours and 30 days' MTA setting time intervals except in the 10-minute interval. Therefore placement of RC over partially set MTA directly might result in low micro-mechanical and/or chemical interaction between the two materials.

a. Pierre Rolland, Merignac France.
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Appendix 2: CONSORT checklist*

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	127
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	127
	2b	Specific objectives or hypotheses	130
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	131
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	
Participants	4a	Eligibility criteria for participants	133
	4b	Settings and locations where the data were collected	132
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	134-135
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	143
	6b	Any changes to trial outcomes after the trial commenced, with reasons	
Sample size	7a	How sample size was determined	131
	7b	When applicable, explanation of any interim analyses and stopping guidelines	
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	132
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	132
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	132
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	135-136

Section/Topic	Item No	Checklist item	Reported on page No
	11b	If relevant, description of the similarity of interventions	
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	143
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	145
	13b	For each group, losses and exclusions after randomisation, together with reasons	146
Recruitment	14a	Dates defining the periods of recruitment and follow-up	
	14b	Why the trial ended or was stopped	
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	146
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	148
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	148
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	155
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	159
Other information			
Registration	23	Registration number and name of trial registry	131
Protocol	24	Where the full trial protocol can be accessed, if available	
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

Appendix 3: Research Ethics Committee approval letter

NHS
Health Research Authority

NRES Committee London - South East
Bristol Research Ethics Committee Centre
Level 3, Block B
Whitefriars,
Lewins Mead,
Bristol
BS1 2NT

Telephone: (0117) 3421382

09 July 2014

Professor Francesco Mannocci
Department of Conservative Dentistry, Guy's Hospital
Floor 25, Tower Wing
London SE1 9RT

Dear Professor Mannocci

Study title:	**Assessment of a new protocol for indirect pulp capping procedures**
REC reference:	**14/LO/0880**
IRAS project ID:	**156456**

Thank you for your letter of 7 July 2014, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a Sub-Committee of the REC. A list of the Sub-Committee members is attached.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Mr Rajat Khullar, nrescommittee.london-southeast@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

<u>Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.</u>

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

A Research Ethics Committee established by the Health Research Authority

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet completed any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as an SSA application(s) has been reviewed. In the meantime no study procedures should be initiated at non-NHS sites.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering letter on headed paper [Cover letter]		07 July 2014
GP/consultant information sheets or letters [GDP letter]	Version 2	07 July 2014
IRAS Checklist XML [Checklist_07072014]		07 July 2014
IRAS Checklist XML		
Other [Good clinical practice]		
Other [Case report form]	1.0	
Other [Avijit Banerjee]		
Other [HTA and consent taking]		
Other [Medical Expert Report: New Dose Report CBCT pulp capping agent]	1	
Participant consent form	1.0	08 May 2014
Participant information sheet (PIS) [PIS]	Version 2	07 July 2014
REC Application Form [REC_Form_07072014]		07 July 2014
Referee's report or other scientific critique report [Peer Review report]		08 May 2014
Research protocol or project proposal [Clinical protocol]	1.0	08 May 2014
Summary CV for Chief Investigator (CI) [Prof. Francesco Mannocci]		
Summary CV for student [Ahmed H. Ali]		08 May 2014
Summary CV for supervisor (student research) [Prof. Francesco Mannocci]		
Summary, synopsis or diagram (flowchart) of protocol in non technical language	1.0	

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

A Research Ethics Committee established by the Health Research Authority

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/LO/0880	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

Wai Yeung

Mr Wai Yeung
Research Ethics Committee (REC) Assistant

pp Professor David Caplin
Chair

Email:nrescommittee.london-southeast@nhs.net

Enclosures:	*List of names and professions of members who were present at the meeting and those who submitted written comments*
	"After ethical review – guidance for researchers"
Copy to:	*Mr. Keith Brennan* *Ms Karen Ignatian, Guy's & St Thomas' Foundation NHS Trust*

Appendix 4: Research and Development approval letter

R&D Site file - Assessment of a New Protocol for Indirect Pulp Capping - 14/LO/0800

Fay Rachel <Rachel.Fay@gstt.nhs.uk>

Mon 7/21/2014 3:44 PM

To:Mannocci, Francesco <francesco.mannocci@kcl.ac.uk>;

Cc:Patel Anjali <Anjali.Patel@gstt.nhs.uk>; Ali, Ahmed <ahmed.ali@kcl.ac.uk>;

Dear Professor Mannocci

Title: Assessment of a New Protocol for Indirect Pulp Capping
REC: 14/LO/0800
PI: Professor Francesco Mannocci
Sponsor: KCL

Thank you for submitting your study to GSTFT R&D Department I am delighted to inform you that NHS Permission has been issued for the above study. We have prepared a site file that will include the R&D approval letter and we will need to meet and explain your responsibilities as an investigator in order to remain compliant under the Research Governance Framework.

Please let me know when would be convenient to meet either at your office or at the R&D offices, 16th floor Tower Wing, Guy's Hospital.

As you may be aware, the Trust is working to achieve the national and local ambition of:

→ 80% studies recruiting their first participant within 30 days
→ 80% of studies recruiting the agreed number of participants within the planned study duration

For your study, the targets are recruiting your first participant by **21/08/2014** and recruiting **120 participants** in total by **the end of the trial.** If you are not able to meet these targets please do contact me to discuss an extension to the end date or other options.

You will need to send by email a monthly report of the recruitment numbers to the studies i.e. the numbers of participants recruited to your studies every month. This reporting is now a Department of Health requirement and the Trust is tasked with gathering data on every active study taking place at the organisation.

The accrual notification should be sent to: R&Drecruitment@gstt.nhs.uk

Stating:

1. The R&D number (RJ112/N) number given to you by the R&D department
2. The REC REF number
3. The Month and year
4. And the number recruited to the study for that month

Please note **Ahmed Ali** will have to have a research passport before he can join the trial – see previous email for details and correspondence from Anjali Patel cc'd in *

If you have any queries throughout your project, please do not hesitate to contact me. Meanwhile, may I wish you success in your project.

Kind Regards,

Rachel

https://outlook.office.com/owa/?viewmodel=ReadMessageItem&ItemID=AAMkADE4NzIjMmFILTEzNzAtNGRkNI04Yzg5LWE0NWE0ZmFIODY0MgBGA... 1/2

Appendix 5: Clinical trial participant's information sheet

Guy's and St Thomas' NHS
NHS Foundation Trust

KING'S College LONDON

Guy's Hospital
Great Maze Pond
London SE1 9RT

PARTICIPANT INFORMATION SHEET

1. **Study title**

"Assessment of a new protocol for indirect pulp capping procedures".

2. **Invitation paragraph**

You are being invited to take part in a research study. Before you decide whether or not to do so, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. *Thank you for reading this.*

3. **What is the purpose of the study?**

Tooth decay is one of the most common diseases in the world. Very often, a patient comes to the dental clinic when experiencing pain that means that the decay is deep and is very close to or has breached the nerve of the tooth. When the decay is very close to the pulp or nerve of the tooth, a procedure called indirect pulp capping (covering) is undertaken in an attempt to save the tooth. This study aims firstly to examine the nerve response (vitality of tooth) to the pulp capping procedure comparing two techniques (two groups), the standard clinical procedure used commonly by general dental practitioner (which includes the removal of decay by naked eye) and a new clinical protocol which includes decay removal with the aid of an operating microscope to improve the visualization of the operating field, to minimize unnecessary removal of healthy tooth structure and to prevent infection of the nerve with bacteria.
The microscope used is an operating microscope which is used routinely by root canal experts and some general dentists and is positioned approximately 30 cm from the mouth of the patient. Therefore you will be assigned to one of the two groups of the study arbitrarily. All other materials used in this study are used routinely by general dentists.
Samples of decayed tooth tissue will be collected using the normal hand instrument (excavator) which dentists used to remove decay from the tooth. This sample collection will add approximately 1 minute to your overall treatment time. There will be no adverse effects from this procedure as the removal of tooth decay is a normal part of dental treatment. The aim of this procedure is to examine in a laboratory the types and spreading of bacteria associated with this disease, so helping to design a

new treatment in the future. All samples will be unidentified and not traceable to the patient.
X-rays are used routinely to assess how close the decay is to the nerve and to see if there are abnormalities around the tooth. However conventional X-rays may not be accurate. A new technique called Cone Beam Computed Tomography (CBCT) has been developed which shows a 3-D image of the tooth and may be more accurate. We aim to compare the images gained from conventional X-rays and images of CBCT to help improve diagnosis and care planning for patients. This study is being carried out in partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) of Mr. Ahmed H Ali.

4. **Why have I been invited?**

We are inviting you to take part in this study because you have deep caries and need the pulp capping procedure as part of your routine care. This makes you suitable for this study. We are going to assign you arbitrarily to one of the two groups of the study according to the technique used.

5. **Do I have to take part?**

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. If you do not take part in the study you will not be required to give a reason and there will be no detriment. This will not affect the standard of care you receive in any way. We will ask your consent for informing your general dental practitioner (GDP) about your participation in this study. If you don't consent to us telling your GDP, you can still stay in the study.

6. **What will happen to me if I take part?**

If you decide to take part in this research your dental care will proceed as normal. Every procedure done in this research study is a routine dental procedure with the exception of CBCT. On the other hand you will have the chance to be treated by specialist dentist.

7. **What do I have to do?**

Patients who agree to take part in this study will be required to attend 4 visits to Guy's Hospital for follow-up of the restorations during an 18 month period free of charge. Should you require other dental treatment, this will be provided at your follow-up visits, free of charge, within the clinical acceptance criteria of Guy's and St. Thomas' Hospitals Foundation Trust (GSTFT).

8. **What is the drug or procedure that is being tested?**

We are not testing any new material under development. The material we are using are already fully authorised and approved for routine clinical use and are available commercially to all dentists. We are trying to find out the beneficial effect of a new operative technique in the procedure of pulp capping which involve the use of operative microscope to improve the visualization of the operator. Moreover cone beam computed tomography (CBCT) has been proved by previous research studies to detect the presence of bony disease around the tooth roots earlier than conventional X-rays. This may help improve our diagnosis and care plan for future patients.

9. **What are the side effects of taking part?**

There are no side-effects of taking part in this study other than those expected from routine dental care.

10. **What are the possible disadvantages and risks of taking part?**

Every exposure to ionising radiation (x-rays) carries a risk. However, due to the low doses of radiation from dental x-rays including CBCT, this risk is negligible. Periapical x-rays are normally taken for routine dental treatment and the effective dose from this conventional x-ray is equal to 0.19% of annual background radiation. This is the same as cosmic radiation exposure on board an aircraft for a 3 hour flight. The additional dose comes from the CBCT scan which is equal to 2.43% annual background radiation and is the same as cosmic radiation on board an aircraft for a 20 hour long-haul flight, e.g. from the UK to Japan or Australia.

11. **What are the possible benefits of taking part?**

There are no advantages to you personally from taking part. If you participate or not, you will still receive appropriate dental care as per the acceptance criteria of GSTFT. The information we get from this study may help us to treat future patients better.

12. **What if there is a problem?**

Questions and Concerns – If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. Please contact: Principle Investigator (Francesco.mannocci@kcl.ac.uk) telephone number (020718881573)

Complaints – If you have a complaint, you should talk to your research doctor who will do their best to answer your questions. If you remain unhappy, you may be able to make a formal complaint through the NHS complaints procedure. Details can be obtained through the Guy's and St Thomas' Patient Advisory Liaison Service (PALS) on 0207 1888188, address: PALS, KIC, Ground floor, north wing, St Thomas' Hospital, Westminster Bridge Road, London, SE1 7EH .

Harm – This trial is co-sponsored by King's College London and Guy's and St Thomas' NHS Foundation Trust. The sponsors will at all times maintain adequate insurance in relation to the study independently. Kings College London, through its own professional indemnity (Clinical Trials) and no fault compensation and the Trust having a duty of care to patients via NHS indemnity cover, in respect of any claims arising as a result of clinical negligence by its employees, brought by or on behalf of a study patient.

13. Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

14. What will happen to the results of the research study?

Results of this research will be published in appropriate dental and scientific journals. No personal information or other information that could be identified as relating to you will be published. You will be informed of the results of the study.

15. Who is organising and funding the research?

King's College London.

16. Who has reviewed the study?

This study was reviewed by NRES Committee London- South East.

17. Summary

You are invited to participate in this study because you have deep caries. This will be cleaned out and you will receive a pulp capping materials covered by permanent filling (tooth-coloured resin composite). During the next follow-up visits your tooth will be examined and the fillings evaluated. You will receive x-rays at your first visit and after one year.

18. Contact for Further Information

For further information please contact:
PhD student: Ahmed H. Ali
King's College London Dental Institute
Biomaterials research group
Floor 17 Guy's Tower

Appendix 6: Clinical trial consent form

Guy's and St Thomas' NHS
NHS Foundation Trust

KING'S College LONDON

Study Number:
Patient Identification Number for this trial:

CONSENT FORM FOR RESEARCH STUDY

Title of Project: "Assessment of new protocol for indirect pulp capping procedures"
Name of Researcher: Prof. Francesco Mannocci, Ahmed H Ali

Please initial the boxes after the statement that you consent or agree with

- I confirm that I have read and understood the information sheet dated **08-05-2014** (version 1.0) for the above study. ☐
- I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
- I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
- I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from Guy's Hospital, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
- I agree to my General dental professional being informed of my participation in the study ☐
- I agree to take part in the above research study. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature

1 CONSENT FORM Version 1.0 08-05-2014

Researcher	Date	Signature

1 copy for patient: 1 copy for researcher site file: 1 (original) to be kept in medical notes.

Appendix 7: Clinical trial case report form

Guy's and St Thomas' NHS
NHS Foundation Trust

KING'S College LONDON

Case Report Form

Baseline Visit

Patient Identification Number/Code:

Date:

Demographics:

Gender	Male (0) ☐	Female (1) ☐	
Date of Birth	Day	Month	Year
Age			
Ethnicity	Caucasian ☐ Hispanic ☐	Black ☐ Other ☐	Asian ☐
Occupation			
Smoking status	Smoker ☐	Non-Smoker ☐	
Pregnancy	Pregnant ☐	Not Pregnant ☐	N/A ☐
Alcohol Consumption			

Medical History*:

Are there any medical conditions to report? Yes* ☐ No ☐ *If "Yes" please describe below:
***Note:** A separate detailed medical history chart is included in the patient's notes.

Dental History:

Presenting Complaint	
History of presenting complaint	Commencement: Location: Type of pain: Incidence: Duration: Initiating/Relieving factors:
Past Dental History	Previous dental treatment: How regularly do you visit your dentist? Preventive protocols do you follow?
Social History	Availability for appointments:
Habits	OH procedures/frequency: Diet: Parafunctional habits:

Full Clinical Oral Examination

Extra-Oral Examination			
	Present	Absent	Comment
Normal Facial Symmetry	☐	☐	
Normal Temporomandibular Joint	☐	☐	
Normal Lips	☐	☐	
Normal Upper Cervical Lymph Nodes	☐	☐	
Normal Submandibular Triangle	☐	☐	
Normal Salivary Glands	☐	☐	
Other, (specify)	☐	☐	

Intra-Oral Examination*			
	Present	Absent	Comment
Normal Mucosa			
Normal Outer and Inner lips			
Normal Gingiva			
Normal Tongue			
Normal Floor of the Mouth			
Normal Hard Palate			
Normal Soft Palate			
Normal Oropharynx			
***Note:** A separate detailed examination chart is included in the patient's notes.			

Clinical Examination of the involved tooth	
Involved tooth number (FDI tooth notation system)	
mICDAS score	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
EPT	
Cold Test	1-Exaggerated response, disappears when stimulus is removed ☐ 2-Exaggerated response, remains for a while after stimulus is removed. ☐ 3- Negative response ☐
Percussion Test	TTP ☐ Not TTP ☐
Palpation Test	Normal ☐ Abnormal ☐
Sinus Tract, fistula, swelling, abscess	Present ☐ Absent ☐
Mobility	0 ☐ 1 ☐ 2 ☐ 3 ☐
Probing Depth (mm)	Buccal ☐ Lingual ☐ Mesial ☐ Distal ☐

***Please fill out the following section if there is more than one tooth involved.**

Clinical Examination of the involved tooth*	
Involved tooth number (FDI tooth notation system)	
mICDAS score	0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐
EPT	
Cold Test	1-Exaggerated response, disappears when stimulus is removed ☐ 2-Exaggerated response, remains for a while after stimulus is removed. ☐ 3- Negative response ☐
Percussion Test	TTP ☐ Not TTP ☐
Palpation Test	Normal ☐ Abnormal ☐
Sinus Tract, fistula, swelling, abscess	Present ☐ Absent ☐
Mobility	0 ☐ 1 ☐ 2 ☐ 3 ☐
Probing Depth (mm)	Buccal ☐ Lingual ☐ Mesial ☐ Distal ☐

Radiographic Examination:

Periapical Date taken:	**CBCT** Date taken:
Report:	Report:

Inclusion Criteria:

Please mark the correct answers to the following questions:

	Yes	No*
1- **Age** Over the age of 16	☐	☐
2- **General health** Good general health with (in the opinion of the investigator) no clinically relevant abnormalities of medical history or oral soft tissue examination.	☐	☐
3- **Carious lesion** A minimum of one carious lesion (occlusal or proximal).	☐	☐
4- **Pulp response** A positive pulp response to electric pulp test or thermal stimulation of the tooth involved.	☐	☐
5- **Compliance** Understands and is willing, able and likely to comply with all the study procedures and restrictions.	☐	☐
6- **Consent** Demonstrates understanding of the study and willingness to participate as evidenced by voluntary written informed consent and has received a signed and dated copy of the informed consent form.	☐	☐
***Note:** *If any of the above questions are answered "No", the subject should be discontinued from the study as a "screen failure" on the Study Conclusion page (p).*		

Exclusion criteria:

Please mark the correct answers to the following questions:

	Yes*	No
1- **Pregnancy** Women who are known to be pregnant or who are intending to become pregnant over the duration of the study.	☐	☐
2- **Condition of the tooth/teeth involved**		
• Clinical symptoms of irreversible pulpitis requiring endodontic treatment.	☐	☐
• The presence of fistulas or swelling.	☐	☐
• Anterior tooth/teeth with aesthetic concerns.	☐	☐
• The presence of radiolucencies or widening of the apical periodontal ligament space.	☐	☐
• External or internal root resorption.	☐	☐
• Mobile tooth/teeth or tenderness to percussion.	☐	☐
3- **Allergy/Intolerance** Known or suspected intolerance or hypersensitivity to the study materials (or closely related compounds) or any of their stated ingredients.	☐	☐
4- **Dental condition** Evidence of gross intra oral neglect.	☐	☐
***Note:** *If any of the above questions are answered "Yes", the subject should be discontinued from the study as a "screen failure" on the Study Conclusion page (p).*		

Fitness and Eligibility to Participate in Study:

In the investigator's opinion, on the basis of the screening assessment and Inclusion and Exclusion criteria at this visit, is the subject eligible and fit to participate in the next part of the study? ☐ Yes ☐ No

Investigator's Signature________________ Date________________

Tooth Eligibility and Randomisation

Pulp exposure after caries affected dentin removal.	☐ Yes* ☐ No
If "Yes" please record size and time taken for haemostasis.	Size: Time taken for haemostasis:
Is the tooth still eligible for inclusion?	☐ Yes ☐ No*
	*If "No" is checked, the subject should be discontinued from the study due to "Protocol deviation" on the Study Conclusion page (p) unless another tooth is eligible for inclusion.
Was the tooth/teeth randomised?	☐ Yes* ☐ No
	*If "Yes" is checked, please complete the following
Date of randomisation	Day Month Year
Randomisation code	

Digital Imaging Procedure (Photography)

Has the subject had digital imaging?	☐ Yes ☐ No

Subject Eligibility:

Has there been any deviation from the protocol since the last visit? ☐ Yes* ☐ No

If "Yes", please record the deviation details on the comments page.

Did any untoward signs/symptoms appear or worsen since the last visit? ☐ Yes* ☐ No

If "Yes", please describe on the comments page.

Is the subject eligible to continue in the study? ☐ Yes ☐ No*

If "No", please continue the Study Conclusion page.

Clinical Examination:

Clinical Examination of the involved tooth	
Involved tooth number (FDI tooth notation system)	
EPT	
Cold Test	1-Exaggerated response, disappears when stimulus is removed ☐ 2-Exaggerated response, remains for a while after stimulus is removed. ☐ 3- Negative response ☐
Percussion Test	TTP ☐ Not TTP ☐
Palpation Test	Normal ☐ Abnormal ☐
Sinus Tract, fistula, swelling, abscess	Present ☐ Absent ☐
Mobility	0 ☐ 1 ☐ 2 ☐ 3 ☐
Probing Depth (mm)	Buccal ☐ Lingual ☐ Mesial ☐ Distal ☐

Digital Imaging Procedure (Photography)

Has the subject had digital imaging?	☐ Yes	☐ No

Comments:

Please indicate any additional information that has not been addressed on the previous case report pages.

Page	Section Heading	Comment

Study Conclusion:

Did the subject complete the entire study? ☐ Yes ☐ No*

*If "No" is marked, please indicate the primary reason below. Please mark only one.

Lost to follow-up	☐	
Protocol deviation	☐	
Withdrawal of Consent	☐	Please specify:
Adverse Event	☐	Please specify:
Other	☐	Please specify:

Was there contact with the subject after the final visit? ☐ Yes ☐ No

If there was contact in relation to this study, please complete the following.

Method of contact: ☐ Telephone ☐ Letter

☐ Other Please specify:

Date of last contact:

Investigator's Signature:

I confirm that I have reviewed all the data collected in this Case Report Form and take responsibility that the information is accurate and complete.

Principle Investigator's Signature:__________________Date:__________________

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